การเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิด เอ ในสัตว์ปีกและสุนัข ในเขตย่างกุ้ง และเนปิดอว์ ประเทศพม่า

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SURVEILLANCE OF INFLUENZA A VIRUSES IN AVIAN AND CANINE SPECIES IN YANGON AND NAY PYI TAW, MYANMAR

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Public Health
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Field of Study	Veterinary Public Health
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วิทยานิพนธ์นี้ประกอบไปด้วยระบบการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในสัตว์ปีกและสุนัขในประเทศพม่า ระบบการเฝ้าระวังเชื้อไวรัสในสัตว์ทั้ง 2 ประเภทประกอบไปด้วยการติดตามระบบการเฝ้าระวังโรคทางไวรัสและทางซีรัม นอกจากนี้ยังมีการวิเคราะห์ข้อมูลทางระบาดวิทยาเพิ่มเติมขึ้นในระบบการเฝ้าระวังในสุนัข ระบบการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในสัตว์ปีกเป็นเวลา 14 เดือนได้ทำการศึกษาในตลาดค้าสัตว์ปีกมีชีวิตในเมืองย่างกุ้งและเมืองนามคาน ประเทศพม่า ตัวอย่างสวอปจำนวนทั้งหมด 1,278 ตัวอย่าง ถูกเก็บจากสัตว์ปีกเพื่อนำไปตรวจหาเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ โดยตัวอย่างดังกล่าวเก็บจากไก่จำนวน 703 ตัวอย่าง เป็ด 380 ตัวอย่าง และจากสิ่งแวดล้อมจำนวน 195 ตัวอย่าง อัตราความชุกของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในสัตว์ปีกคิดเป็น 5.71% (73/1,278) โดยสามารถแยกเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ สายพันธุ์ H9N2 ได้จำนวน 3 ตัวอย่างจากไก่ในตลาดค้าสัตว์ปีกมีชีวิตในเมืองนามคาน โดยผลจากการวิเคราะห์ทางพันธุกรรมพบว่าเชื้อไวรัสดังกล่าวเป็นเชื้อไวรัสไข้หวัดใหญ่ชนิดรุนแรงต่ำ และมีลักษณะทางพันธุกรรมใกล้เคียงกับเชื้อไวรัสสายพันธุ์ H9N2 ที่พบในประเทศจีนในช่วงปี 2013-2015 การติดตามระบบการเฝ้าระวังโรคทางซีรัมในสัตว์ปีกเป็นเวลา 8 เดือน ตั้งแต่เดือนกุมภาพันธ์ถึงกันยายน 2016 ได้ทำการศึกษาในตลาดค้าสัตว์ปีกมีชีวิตในเมืองย่างกุ้ง โดยตัวอย่างซีรัมจำนวนทั้งหมด 621 ตัวอย่าง ถูกเก็บจากไก่และเป็ดจำนวน 489 และ 132 ตัวอย่างตามลำดับ อัตราความชุกของแอนติบอดีเชื้อไวรัสไข้หวัดใหญ่ชนิดเอคิดเป็น 12.8% (80/621) จากตัวอย่างทั้งหมดจำนวน 80 ตัวอย่างที่ให้ผลบวกต่อ ELISA พบว่าเป็นเชื้อไวรัสไข้หวัดใหญ่ชนิดเอ สายพันธุ์ H5N1 จำนวน 9 ตัวอย่าง และเป็นสายพันธุ์ H9N2 จำนวน 15 ตัวอย่าง ระบบการเฝ้าระวังเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในสุนัขเป็นเวลา 12 เดือนได้ทำการศึกษาในเมืองย่างกุ้งและเนปิดอว์ เดือนมิถุนายน 2014 ถึง พฤษภาคม 2015 อัตราความชุกของเชื้อไวรัสไข้หวัดใหญ่สุนัขในสุนัขคิดเป็น 21.2% (43/203) จากผลการวิเคราะห์หาความสัมพันธ์เกี่ยวกับการติดเชื้อไวรัสไข้หวัดใหญ่สุนัข พบว่าอัตราความชุกของการติดเชื้อไวรัสไข้หวัดใหญ่สุนัขมีความสัมพันธ์อย่างมีนัยทางสถิติกับปัจจัยต่างๆ ได้แก่ อาการทางระบบทางเดินหายใจ (p=0.004; OR=2.92; 95% CI=1.387 – 6.143) คะแนนความสมบูรณ์ทางร่างกายที่ต่ำ (p=0.015; OR=3.02; 95% CI=1.319 – 6.895) ฤดูกาลที่ทำการเก็บตัวอย่าง (p=0.001; OR=8.31; 95% CI=3.224 - 21.426) และ การทำวัคซีนไวรัสไข้หวัดใหญ่ (p=0.008; OR=9.69; 95% CI=1.283 - 73.24) และจากผลการวิเคราะห์หาความสัมพันธ์ของแอนติบอดีของเชื้อไวรัสไข้หวัดใหญ่สุนัข พบว่าอัตราความชุกของแอนติบอดีของเชื้อไวรัสไข้หวัดใหญ่สุนัข มีความสัมพันธ์อย่างมีนัยทางสถิติกับปัจจัยต่างๆ ได้แก่ อาการทางระบบทางเดินหายใจ (p=0.009; OR=3.16; 95% CI= 1.291 – 7.735) และฤดูกาลที่ทำการเก็บตัวอย่าง (p=0.001; OR=4.583; 95% CI= 1.804 – 11.645) นอกจากนี้การศึกษานี้ยังพบว่าอาการทางระบบทางเดินหายใจและคะแนนความสมบูรณ์ทางร่างกายที่ต่ำมีความสัมพันธ์อย่างมีนัยสำคัญทางสถิติต่อผลตรวจทาง RT-PCR และ ELISA ($p<0.05$) จากผลการศึกษาครั้งนี้แสดงให้เห็นถึงการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอของสัตว์ในประเทศพม่าที่มีการกระจายตัวและมีความซับซ้อนมากขึ้น ระบบการเฝ้าระวังและการตรวจวิเคราะห์ลักษณะทางพันธุกรรมของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอเพื่อตรวจสอบการอุบัติใหม่ของการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในกลุ่มประชากรสัตว์ควรกระทำอย่างต่อเนื่อง ทั้งในระดับท้องถิ่น ระดับภูมิภาค และระดับประเทศ อีกทั้งการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอของประชาชนในประเทศพม่าควรได้รับความสนใจและให้ความสำคัญทางสาธารณสุขมากขึ้น อีกทั้งยังควรมีการเฝ้าระวังการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอระหว่างคนและสัตว์ ความร่วมมือระหว่างหน่วยงานต่างๆถือว่าเป็นส่วนสำคัญเพื่อช่วยลดการแพร่กระจายของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอภายในภูมิภาค นอกจากนี้ข้อมูลที่ได้จากการศึกษาครั้งนี้ยังสามารถนำไปใช้เป็นข้อมูลในการวางแผนเพื่อทำการศึกษาและควบคุมการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในประเทศพม่าได้ต่อไปในอนาคต

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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THANT NYI LIN: SURVEILLANCE OF INFLUENZA A VIRUSES IN AVIAN AND CANINE SPECIES IN YANGON AND NAY PYI TAW, MYANMAR. ADVISOR: PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 143 pp.

This dissertation contains influenza surveillance programs conducted in two animal species, poultry and dogs, in Myanmar. In poultry, the study included virological and serological influenza surveillances. In dogs, the study included virological and serological influenza surveillances as well as epidemiological analysis (association study). In poultry, 14-month influenza surveillance program was conducted in Live-bird Markets (LBM) in Yangon and Namkham, Myanmar. One thousand two hundred and seventy-eight (1,278) swab samples were collected from chickens (n=703), ducks (n=380), and environments (n=195) and tested for influenza A virus (IAV). The occurrence of IAV in poultry was 5.71% (73/1,278). Interestingly, three IAV subtype H9N2 were isolated from chicken in Namkham, upper Myanmar. Genetic analysis of the IAV-H9N2 revealed that the viruses posed low pathogenicity characteristics and closely related with IAV-H9N2 isolated from China during 2013-2015. For serological surveillance in poultry, 8-month influenza surveillance program was conducted in Yangon LBM during February to September 2016. Six hundred twenty-one (621) serum samples were collected from chickens (n=489) and ducks (n=132). The occurrence of IAV antibodies was 12.8% (80/621). Out of 80 ELISA-positive samples, 9 samples were subtyped as H5N1 and 15 samples were subtyped as H9N2 by HI test. In dogs, 12-month influenza surveillance program was conducted in Yangon and Nay Pyi Taw areas, Myanmar. The study was conducted in dogs in four townships of Yangon and Nay Pyi Taw during June 2014 to May 2015. The occurrence of canine influenza A virus (CIV) in dogs was 21.2% (43/203) by real-time RT-PCR. It is noted that no virus could be isolated from dogs. For serological surveillance in dogs, the occurrence of CIV antibodies was 11.8% (24/203). In association analysis of CIV infection in dogs, the results showed that the occurrence of CIV infection in dogs was statistically significant associated with respiratory symptoms (p=0.004; OR=2.92; 95% CI=1.387 – 6.143), poor body condition score (p=0.015; OR=3.02; 95% CI=1.319 – 6.895), season of sampling (p=0.001; OR=8.31; 95% CI=3.224 - 21.426), and vaccination against influenza viruses (p=0.008; OR=9.69; 95% CI=1.283 - 73.24). In association analysis of CIV antibodies, the results showed that the occurrence of CIV antibodies was statistically significant associated with respiratory symptoms (p=0.009; OR=3.16; 95% CI= 1.291 – 7.735) and season of sampling (p=0.001; OR=4.583; 95% CI= 1.804 – 11.645). In this study, the presence of respiratory symptoms and poor body condition scores were statistically significant correlated with test results of RT-PCR and ELISA ($p<0.05$). In conclusion, this dissertation reported the molecular characteristics of IAV H9N2 in Myanmar, and epidemiological information of CIV infection in dogs in Myanmar. Our findings provide insight information regarding influenza A virus infections in animals. Therefore, routine surveillances and genetic characterization of IAVs in animals should be conducted at local, regional and national levels to monitor the emergence of IAVs in animal population. Public health awareness of IAV infection in humans in Myanmar should be provoked to a higher extent and surveillance of IAV infection at human-animal interface is deemed necessary. Collaborative works are needed between authorities to minimize the spread of IAVs across the regions. Findings and recommendations from this study will be useful for prevention and control measures of IAV infections in Myanmar in the future.

Department: Veterinary Public Health Student's Signature .....
Field of Study: Veterinary Public Health Advisor's Signature .....
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LIST OF ABBREVIATIONS

95% CI	95% confidence interval
AGID	Agar Gel Immunodiffusion
AI	Avian influenza
AIV	Avian influenza virus
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	Complementary deoxyribonucleic acid
CIV	Canine influenza virus
CS	Cloacal swab
Ct	Cycle threshold
dNTP	Deoxynucleotide triphosphates
ELISA	Enzyme-linked Immunosorbent Assay
EV	Environmental swab
FAO	Food and Agriculture Organization
HA	Hemagglutinin
HI	Hemagglutination inhibition
HPAI	Highly pathogenic avian influenza
IAV	Influenza A virus
LBM	Live-bird market
LBVD	Livestock Breeding and Veterinary Department
LPAI	Low pathogenic avian influenza
M	Matrix
NA	Neuraminidase
Neg	Negative
NK	Namkham
NP	Nucleoprotein
NPT	Nay Pyi Taw
NS	Nasal swab

NS	Nonstructural protein
OIE	World Organization for Animal Health
OR	Odds ratio
OS	Oropharyngeal swab
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PCR	Polymerase Chain Reaction
Pos	positive
RBC	Red blood cell
RNA	Ribonucleic acid
rRT-PCR	Real-time Reverse Transcription Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
WGS	Whole Genome Sequencing
WHO	World Health Organization
YGN	Yangon

CHAPTER I INTRODUCTION

1.1 Importance of this study

Influenza, caused by influenza virus, is an infectious disease of respiratory infections in animals and humans. Influenza virus is classified into four different types depending on the NP protein, namely influenza A, B, C and D viruses (Webster et al., 1992). Influenza A is responsible for seasonal and epidemic influenza outbreaks in a variety of animal species such as poultry, birds, pigs, horses, dogs and cats, as well as in other mammalian species and humans. Influenza B is related to mild infections in humans and seals, while influenza C is reported in humans with little or no medical significance (Zambon, 1999). Influenza D virus was detected in swine and cattle (Hause et al., 2014; Ducatez et al., 2015; Chiapponi et al., 2016).

For influenza A viruses, viral infection usually depends on the animal species due to species-specific characteristics of the virus. However, interspecies transmission could be observed among different animal species. From the public health point of view, influenza A viruses pose serious threats to humans, since the viruses are of pandemic potential. All pandemic and the vast majority of epidemic outbreaks in the history were caused by influenza A viruses (Webster et al., 1992). The first report of pandemic influenza outbreak, known as “Spanish Flu”, was caused by influenza A virus subtype H1N1. Forty years later, influenza A virus subtype H2N2 caused pandemic influenza outbreak, and it was followed by H3N2 pandemic

influenza outbreak “Hong Kong flu” in 1968. The recent pandemic influenza outbreak in 2009 was caused by influenza A virus subtype H1N1 (Guan et al., 2010). Influenza pandemics can occur whenever a new strain of virus is transmitted from animals to humans, and subsequently from human to human. The influenza virus with pandemic potential can emerge from reassortment and mutation process of the viruses that can be virulent and pose high risk to human health (Garten et al., 2009).

Influenza A virus infections in humans are mostly originated from the exposure of the virus from the reservoirs to domestic animals (Olsen et al., 2006). For avian Influenza (AI), infection in humans takes place through the direct contact with infected poultry (Cowling et al., 2013). Some avian influenza A viruses such as subtypes H5N1 and H7N9 are reported to infect humans with high mortality. Avian influenza is not only an important zoonotic disease, but also is an economic concern due to production loss. Hundreds of millions of poultry have been culled and died from avian influenza in the past three decades in Asia, Europe and USA (Lupiani and Reddy, 2009).

At present, the highly pathogenic avian influenza A virus subtype H5N1 (HPAI H5N1) is already panzootic in poultry and has been identified in 77 countries. The public health impact of HPAI H5N1 is high due to fatal outcome of the infection in humans (Peiris et al., 2007; OIE, 2016a). Since the first report of H5N1 infection in humans in May 1997, the H5N1 infection in humans spread across geographical regions and continents, originating from east and southeast Asia. Series of reports

have been emerged from human H5N1 infections in Asia, Europe, and Africa (Lai et al., 2016). As of November 2016, a total of 856 H5N1 cases had been documented in humans with 53% (452) deaths (WHO, 2016).

For canine influenza, influenza A subtype H3N2 and H3N8 can infect canine species. The viruses are antigenically different from the virus subtypes circulating in humans. Up to date, there is no report related to the transmission of influenza virus from dogs to humans. However, H3 subtypes are highly adaptable and can be evolved in humans, and therefore the risk of canine influenza in humans is possible (Harder and Vahlenkamp, 2010). Canine influenza is important because dogs are considered to be of zoonotic potential and are mostly in close contact with humans (Nelson and Holmes, 2007; Ozawa and Kawaoka, 2013; Chen et al., 2015).

Despite the lack of evidence of direct transmission of CIV from dogs to humans, close contact between dogs and humans is a factor to be aware of in public health. In addition to H3N2 and H3N8 subtypes, experimental studies demonstrated the presence of sero-conversion and shedding of avian originated H5N1 virus in dogs (Giese et al., 2008). Influenza A (H6N1) virus in dogs was genetically similar to novel influenza A (H6N1) virus that infected human in Taiwan (Yan et al., 2014; Lin et al., 2015). A novel H3N1 virus emerged in dogs through the reassortment between H3N21 and pandemic H1N1 viruses (Song et al., 2012). Therefore, an additional concern indicates that dogs may be acting as a mixing vessel and become a new source of novel influenza A virus to humans.

From the public health point of view, avian influenza is always a significant threat (Katz et al., 2009; Su et al., 2015). Growing attention is paid through human respiratory infections (Kalthoff et al., 2010; Poovorawan et al., 2013). Many organizations, including FAO (Food and Agriculture Organization), OIE (Office Internationaledes Epizooties) and WHO (World Health Organization), are working in collaboration to reduce public health impacts of avian influenza (Chmielewski and Swayne, 2011). Avian influenza is also an "OIE List A" disease and a topic of discussion among medical profession (Institute of Medicine Forum on Microbial Threats, 2005).

In Myanmar, there are limited reports published on the outbreaks of avian influenza. The information regarding the avian influenza is still insufficient, with only H5N1, H5N6 and H9 subtypes identified. Moreover, no report has ever been made on canine influenza. Therefore, in this study, surveillance of influenza A virus in poultry and dogs was carried out in Myanmar to determine the occurrence and genetic diversity of influenza A viruses in poultry and dogs in Myanmar.

1.2 Questions of study

1. What are the occurrence and subtypes of influenza A viruses circulating in poultry and dogs in Myanmar?
2. What are the genetic characteristics and diversity of influenza A viruses isolated from avian and canine species in Myanmar?

3. What are the frequency distribution and associated factors in serology of canine influenza infection among dogs of different ages in Myanmar?

1.3 Objectives of study

1. To determine the occurrence of influenza A viruses in poultry and dogs in Myanmar from 2014 to 2016.
2. To reveal the genetic characteristics and diversity of avian and canine influenza viruses isolated from Myanmar.
3. To investigate the frequency distribution and associated factors in serology of canine influenza infection in dogs from Myanmar.

This dissertation is divided into 6 chapters. Chapter 1 is the overview and global importance of influenza A viruses, focusing on avian and canine influenza. Chapter 2 is the review of literature relevant to influenza A viruses in avian and canine species. Chapter 3 explains the methodologies applied in this study. Chapter 4 presents the results from this study. Chapter 5 is the discussion on the results of this study, and chapter 6 provides conclusion and recommendation from this study.

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CHAPTER II LITERATURE REVIEW

2.1 Influenza A viruses (IAV)

2.1.1 Ecology of influenza A viruses

Influenza viruses belong to the family Orthomyxoviridae that consists of 6 genera: influenza A, influenza B, influenza C, Isavirus, Thogotovirus and Quaranjavirus (Presti et al., 2009; McCauley et al., 2012). Influenza viruses are classified according to their antigenic properties in nucleoprotein (NP) and matrix (M) protein (Webster et al., 1992). Among them, Influenza A viruses are clinically most significant (Cox and Subbarao, 2000).

Influenza A viruses are further divided into multiple subtypes based on their antigenic relationship between hemagglutinin (HA) and neuraminidase (NA) glycoproteins. To date, 18 HA (H1-H18) and 11 NA (N1-N11) subtypes have been recognized. Different subtypes were discovered from different species. Subtype associations seems to be more rigid in mammals, although no clear association between host range and HA subtype is observed. Aquatic and wild birds are natural reservoirs of all influenza A virus subtypes (Webster et al., 1992; Olsen et al., 2006), except H17N10 and H18N11 subtypes, which were newly discovered from bats (Tong et al., 2013). The influenza viruses from bats have not been identified in any other animal species and their transmission status is still unknown. In contrast, influenza viruses from natural reservoirs can infect all animal species and humans. Interspecies

transmission of influenza viruses have been observed among different animal species (Medina and Garcia-Sastre, 2011; Freidl et al., 2014; Short et al., 2015; Joseph et al., 2016) (Figure 1).

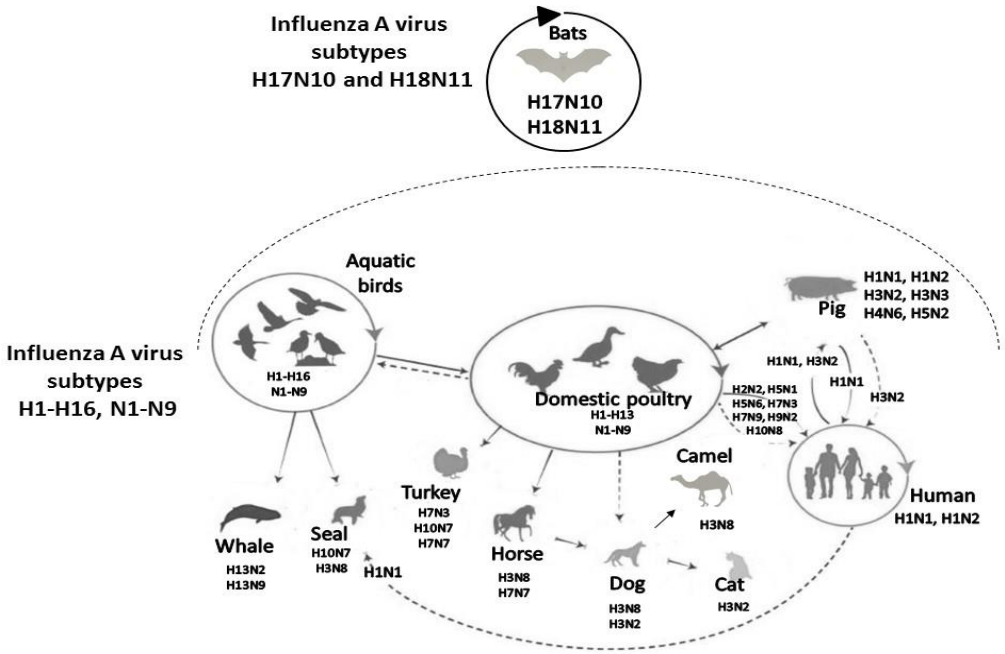

Figure 1: Interspecies transmission of influenza A viruses (modified from Joseph et al., 2016

Clinical symptoms associated with influenza A virus infection can vary depending on the genetic properties of the viruses and species of the host infected. In shorebirds and waterfowls, infection is asymptomatic. Significant morbidity and mortality are observed in domestic animal species and humans (Cox and Subbarao, 2000; Isoda et al., 2006; Taubenberger and Morens, 2008). Seasonal influenza virus infections are huge burden of disease. Constant changes in genetic properties

produce repeated infections in hosts and reviewing of flu vaccine composition is necessary each year. The costs of annual medication, hospitalization, and vaccination results in massive economic blow (Molinari et al., 2007; Uhart et al., 2016).

2.1.2 Morphology of influenza A viruses

Influenza A viruses are negative-sense single-stranded RNA viruses that belong to the family Orthomyxoviridae. The IAV is enveloped and spherical in shape, with the size ranging from 80 to 120 nm. The outer lipid membrane layer of the IAV is derived from the plasma membrane after the virus is propagated in the host. Three glycoproteins (HA, NA, and M2) are embedded in the outer surface of the membrane and M1 (matrix protein) was attached to the membrane from the inner side. Outer surface of the envelope is occupied by spike-like projections of HA proteins and mushroom-like projections of NA proteins. Small M2 proteins on the surface of membrane form ion channels. Inside the envelop, the matrix protein M1 encloses the virion core, inside which the NP proteins and RNA polymerase complex (PB1, PB2, PA proteins) are embedded (Webster et al., 1992; Nayak et al., 2009).

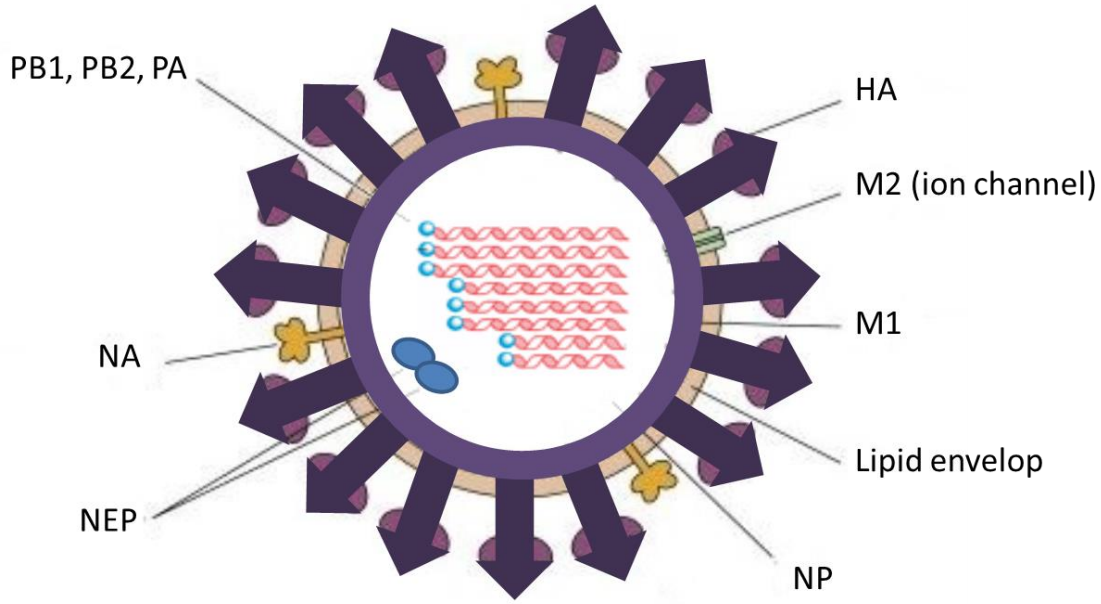

Figure 2: Structure of influenza A virus (Modified from Medina and Garcia-Sastre, 2011)

The influenza virus has a genome of eight RNA segments that encode for nine structural proteins (PB1, PB1-F2, PB2, PA, HA, NA, NP, M1 and M2) and two non-structural proteins (NS1 and NS2) (Table 1). Among these, surface glycoproteins HA and NA are the key components of influenza virus infection. HA protein is responsible for binding the virus to the cell and NA protein is responsible for the release of progeny virions. Influenza A viruses are evolutionarily dynamic with high mutation rates of ($1x10^{-3}$ to $8x10^{-3}$). The changes of amino acid sequences in HA and NA glycoproteins can promote antigenicity of the virus. These properties provide advantages for influenza A viruses to conquer pre-existing immunity in the host and the influenza A viruses can become adapted (Rott, 1992; Webster et al., 1992).

Table 1: Function of gene segments of influenza A viruses

	Protein	Length	Function	References
1	PB2	2,341	Binding host-cell pre-mRNA	(Kapoor and Dhama, 2014)
2	PB1	2,341	Viral mRNA elongation for active binding	(Cobbin et al., 2014)
3	PA	2,233	Cleavage of host pre-mRNA Initiation of transcription	(Kapoor and Dhama, 2014)
4	HA	1,778	Attachment of virus to the host cells	(Mineev et al., 2013)
5	NP	1,565	Template for transcription RNA binding	(Portela and Digard, 2002)
6	NA	1,413	Release of new virus from the host cells	(Cheng et al., 2012)
7	M1	1,027	Enclosing the virion Export of newly formed vRNP Assembly and budding	(Kapoor and Dhama, 2014)
	M2		Acidification of the virion for uncoating of the viral particles	(Schnell and Chou, 2008)
8	NS1	890	Inhibition of pre-mRNA splicing Blockage of mRNA nucleocytoplasmic transport	(Hale et al., 2008) (Fortes et al., 1994)
	NS2 (NEP)		Viral ribonucleocapsids exportation Bridging viral ribonucleoproteins and the nuclear pore complex	(O'Neill et al., 1998)

2.1.3 Genetic reassortment

Genetic materials are constantly changing in the genes of influenza viruses. Due to the segmented genome, influenza viruses can undergo reassortment between different subtypes. In general, the influenza A viruses are closely related to each other and usually share the same antigenic properties despite the reassortment. However, over a long period, these genetic changes accumulate and result in the viruses that are antigenically different and unrecognizable by the immune system. There are two common types of genetic reassortment: antigenic shift and drift (Webster et al., 2013).

2.1.4 Antigenic shift and drift

In antigenic drift, genetic materials are changed through the replication process. During replication, HA and NA genes that encode viral surface antigen undergo mutation. After repeated numbers, these encoding proteins become immensely different from their origin that the antibodies in host cannot neutralize them. In antigenic shift, the change is more abrupt and profound that it can result in new hemagglutinin and/or neuraminidase proteins in the virus. Antigenic shift takes place when the host is infected with two or more different strains of influenza A viruses. New influenza A virus subtypes evolved from the antigenic shift can be so different from the existing IAVs that the hosts have little or no antibodies to protect against the new virus. Antigenic shift happens less frequently while antigenic drift is

taking place all the time (Zambon, 1999; Rabadan and Robins, 2007). The antigenic shift and drift are important because all the pandemic influenza viruses were evolved from the genetic reassortment. Influenza pandemics can occur whenever a new strain of virus, a novel virus, is transmitted from animals to human population in which no known immunity exists (Guan et al., 2010).

Table 2: Influenza A virus subtypes in humans and domestic animal species (Modified from Freidl et al., 2014; Kapoor and Dhama., 2014a; Lin et al., 2015; Xie et al., 2016)

Host	Detected influenza A virus subtypes
Human	H1N1, H2N2, H3N2, H4N8, H5N1, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7, H10N8
Horse	H3N8, H7N7
Pig	H1N1, H1N2, H1N7, H2N3, H3N1, H3N2, H4N6, H9N2
Dog	H1N1, H3N1, H3N2, H3N8, H5N1, H5N2, H6N1
Cat	H3N2, H5N1
Fowl	16 HA (1–16) and 9 NA (1–9) subtypes
Poultry	13 HA (1-13) and 9 NA (1-9) subtypes

2.2 Avian influenza viruses (AIV)

2.2.1 Ecology of avian influenza viruses

Avian influenza viruses (AIV) are influenza A viruses that can infect all kinds of birds species (Alexander, 2007). AIV can also infect other animal species and humans (Webster et al., 1992; Proenca-Modena et al., 2007). There are 16 Hemagglutinin (H1-H16) subtypes and 9 Neuraminidase (N1-N9) subtypes in the name of avian influenza. The HA and NA subtypes of avian influenza viruses can assort into any possible combination, and therefore 144 subtypes can exist. Many of the combinations are found in natural reservoir species, from which all 16 HA subtypes have been discovered. Certain subtypes show species predilection. For example, H13 and H16 are more commonly observed in gulls (Munster et al., 2007). However, all avian species have the same receptor binding site "α-2,3-linked sialic acid" and many avian species are susceptible to avian influenza virus infections. Although airborne transmissibility is possible, the principal mode of transmission is via the faecal-oral route (Herfst et al., 2014). Domestic poultry such as chickens and quails are not natural hosts of influenza A viruses, but they may serve as reservoirs when viruses become adapted in them (Spackman, 2014).

2.2.2 Epidemiology of avian influenza

The earliest date of the appearance of avian influenza, initially named as "fowl plague", was in 1878. However, fowl plague represented both avian influenza and Newcastle viruses, which were unidentifiable those days. The first confirmation of the avian influenza was in 1959 when the influenza A virus subtype H5N1 was isolated from chicken in Scotland. Since then, avian influenza have been documented and become endemic in many countries (Alexander, 2000, 2007; Lupiani and Reddy, 2009). Today avian influenza is reported worldwide (OIE, 2016c). Phylogenetically, all the avian influenza viruses distributed over the world are the descendants of either of the two primary lineages: Eurasian and American lineages (Olsen et al., 2006; Wille et al., 2011).

2.2.3 Highly pathogenic and low pathogenic avian influenza (HPAI and LPAI)

Despite several subtypes classified, avian influenza viruses can be simply classified into two groups, highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), depending on their virulence. HPAI is characterized by severe illness and rapid death, with mortality as high as 100% in poultry. HPAI is strictly attributed to H5 and H7 subtypes (Alexander, 2000). However, not all viruses of H5 and H7 subtypes are HPAI. Most of them are detected as LPAI (Wahlgren, 2011). Moreover, clinical severity of HPAI viruses can vary depending on the strain of the virus and infected bird species. Ducks showed higher resistance to avian

influenza viruses when compared with chickens (Alexander et al., 1986). Ducks are capable of initiating cytokine activation faster than chicken, followed by cellular response (Cornelissen et al., 2013). LPAI viruses are not virulent and cause only mild illness in birds. However LPAI can undergo reassortment and evolve into HPAI (Suarez et al., 2004; Pasick et al., 2005). Different clinical presentations between HPAI and LPAI are compared in table 3.

The HA protein is the determinant of pathogenicity of avian influenza viruses. The infection can be either HPAI or LPAI, depending on the motif of cleavage site sequence in HA protein. In HPAI, multiple basic amino acids are present at the HA0 cleavage sites, whereas in LPAI only a single arginine is present at the cleavage site of HA protein (Rott, 1992; Wood et al., 1993). The biological difference between HPAI and LPAI is that HPAI cause a systemic infection, while LPAI is localized to respiratory and intestinal tract infections. For gross differentiation, an influenza A virus can be classified as HPAI if six or more of eight 4- to 8-week-old susceptible chicken die within 10 days after intravenous inoculation with 0.2 ml of a 1/10 dilution infective allantoic fluid (OIE, 2016b). In humans, the pathogenicity of HPAI and LPAI viruses, mostly H5, H7, and H9 subtypes, can range from mild to severe infection to death (Richard et al., 2014; Reperant et al., 2016).

Table 3: Comparison of clinical presentations between HPAI and LPAI in poultry (modification from Horimoto and Kawaoka, 2005; Spackman, 2014)

Difference	HPAI	LPAI
Subtypes	H5 and H7	H1 – H16
Source	Poultry and wild bird	Poultry
Type of infection	Systemic	Localized
Prominent feature	Hemorrhage wattles and combs swollen	Lethargy, depression, decreased feed and water
Virulence	Severe and lethal	mild or asymptomatic
mortality rate	High (up to 100%)	< 5%
Cut-off value	≥ 75% mortality rate	< 75% mortality rate
Cleavage	Multiple basic amino acids	Not multiple
Transmission to human	Less likely	More likely

2.2.4 Avian influenza in Myanmar

In Myanmar, eight major outbreaks of highly pathogenic avian influenza virus subtype H5N1 (HPAI H5N1) have been reported since 2006. The first outbreak was reported in upper Myanmar in March 2006. More than half a million birds from 545 farms in 13 townships were killed. The second outbreak occurred in lower Myanmar in February 2007. The outbreak was reported in ten townships and approximately 100,000 birds from 76 farms were killed. In late 2007, two consecutive outbreaks of HPAI H5N1 occurred in Shan state, a few months after the outbreak of HPAI H5N1 in Guangdong, China. More than 300,000 birds died. During February and March of

2010, the fourth outbreak occurred simultaneously in both upper and lower Myanmar. Sixteen thousand birds from 31 farms were reported dead (Mon et al., 2012). The fifth outbreak was reported in January 2011 in Sagaing and Rakhine state, in which approximately 130,000 birds from four townships were affected. In 2012, the sixth outbreak occurred in Sagaing (upper Myanmar) and Bago (lower Myanmar) regions affecting more than 30,000 birds from four farms. The seventh outbreak of HPAI H5N1 occurred in Sagaing region (upper Myanmar) in February 2015. Approximately 350,000 poultry from 171 farms were culled. In April 2016, the latest outbreak was reported in Sagaing region of upper Myanmar, the same place where the seventh outbreak took place. An approximate of 130,000 birds were destroyed[1].

Apart from HPAI H5N1, IAV subtype H5N6 viruses have been occasionally detected in poultry through routine surveillances in Myanmar. However, no genetic information is available. In 2013, IAV subtype H9 viruses were identified from poultry swab samples collected from border areas near China in northeastern Shan state. The detail information of eight HPAI H5N1 outbreaks in Myanmar is shown in Figure 3 and Table 4.

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[1] Data from Livestock Breeding and Veterinary Department (LBVD), Myanmar

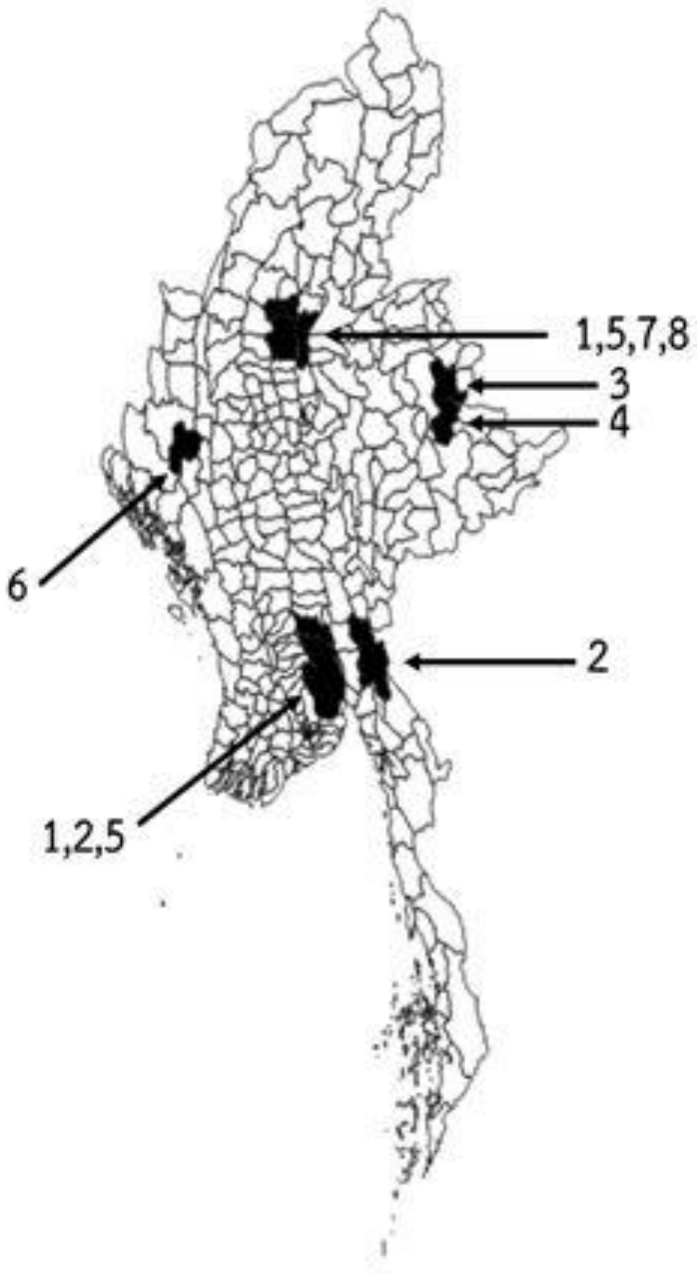

Figure 3: Distribution of eight HPAI H5N1 outbreaks in Myanmar (information related to each outbreak are described in table 4)

2.2.5 Influenza surveillances in border areas in Myanmar

Since Myanmar is bordered with China, which has been regarded as an epicenter for the emergence of novel influenza A viruses (Guan and Smith, 2013; Su et al., 2015), national contingency plans have been established to protect the possible spread of influenza A viruses in Myanmar. Accordingly, surveillances have been carried out in poultry in northeastern Shan state, border areas adjacent to China. Through the surveillances, IAV subtype H9 viruses were identified from poultry in northeastern Shan states in 2013. The viruses showed high genetic similarity with IAV subtype H9N2 viruses recovered from China during 2012 [2].

[2] Annual Conference of Myanmar Veterinary Association, Yangon, 27th – 28th December, 2014

Table 4: Epidemiological data of HPAI H5N1 outbreaks in Myanmar

No	Duration	Township	District	Province	Species	Losses
1	9 Mar – Apr 2006	13 townships	Amarapura Monywa Shwebo	Mandalay Sagaing Sagaing	Chicken, Quail	545 farms, 660,00 birds
2	28 Feb – Mar 2007	10 townships	Yangon Tharyawaddy Mawlamyaing	Yangon Bago Mon	Chicken, Quail, Duck	76 farms, 110,00 birds
3	19 Oct – Dec 2007	2 townships	Kyaing Tong	Shan	Chicken, Duck	30,000 birds
4	3 Feb – Mar 2010	3 townships	Mingalrdon Monywa	Yangon Sagaing	Chicken	31 farms, 16,000 birds
5	16 Jan – Mar 2011	4 townships	Shwebo Rakhine	Sagaing Rakhine	Chicken, Quail, Duck	195 farms, 124,000 birds
6	24 Feb – Mar 2012	2 townships	Chaung U Taunggu	Sagaing Bago	Chicken	4 farms, 30,000 birds
7	21 Feb – Mar 2015	1 township (Poultry zone	Monywa	Sagaing	Chick, Quail	171 farms, 350,000 birds
8	13 Apr – Jun 2016	1 township (Poultry zone)	Monywa	Sagaing	Chicken, Quail	123 farms, 130,000 birds

2.2.6 Live-bird markets (LBM) in Myanmar

Live-bird markets (LBM) are common in South East Asian regions including Myanmar. LBM are usually places for the selling of poultry and processing of poultry meat. On most occasions, slaughtering of the poultry, plucking of the feathers, and chopping of the meat are all accomplished in live-bird markets. Therefore, it is very likely that live-bird markets harbor pathogens in the presence of poor sanitation and improper disposal. In general, LBM are heavily contaminated with pathogens and they are the major source of influenza A viruses. The exchange of genetic materials can take place among different bird species in LBM (Indriani et al., 2010; Kang et al., 2015). Likewise, the disseminating viruses in LBM can transmit to humans (Indriani et al., 2010; Chen et al., 2014b). Several studies already revealed that live-bird markets were associated with the influenza virus infections in humans (Guan et al., 1999; Li et al., 2003; Chen et al., 2013).

2.3 Canine influenza viruses (CIV)

2.3.1 Ecology of canine influenza viruses

Canine influenza viruses (CIV) are influenza A viruses that cause respiratory infection in dogs. Clinical presentations of CIV in dogs include sneezing, fever, persistent cough, and nasal discharges. Severe pulmonary lesions, characterized by necrosis and inflammation of upper and lower respiratory tract can also be seen (Song et al., 2008). In severe cases, hemorrhagic pneumonia will develop and

infection can become fatal (Dubovi and Njaa, 2008). Infected dogs shed virus nasally, but not in the feces. Transmission is by direct contact. Transmission via nasal droplets was proved under experimental conditions (Song et al., 2008). Inter-species transmission of canine influenza virus subtype H3N2 exists among dogs and cats. However, transmission occurs only from dog to cat, not from cat to dog. Intra-species transmission is observed in dogs, cats and ferret (Yamanaka et al., 2009; Kim et al., 2013).

2.3.2 Epidemiology of canine influenza

CIV has two distinct lineage groups: 1) Asian H3N2 lineage originated from avian species and 2) North American H3N8 lineage originated from equine species (Lee et al., 2016). Canine influenza subtype H3N8 was first detected in dogs in Florida in 2004. It was followed by a series of influenza cases in dogs throughout the year 2005, and canine influenza was considered endemic in USA. The canine influenza virus H3N8 was first isolated from the lungs tissue of dogs. The genetic analysis revealed that the isolated H3N8 virus was closely related to influenza virus subtype H3N8 in horse (Crawford et al., 2005). It is a single trans-species transmission from horse to dog, and no reassortment was observed in any of 8 gene segments (Gibbs and Anderson, 2010).

Canine influenza virus subtype H3N2 was first isolated in Korea in 2007 (Song et al., 2008). Although canine influenza virus subtype H3N2 was first detected in

2007 (Song et al., 2008), serological analysis of previously collected sera suggested that the H3N2 viruses could have been circulating in dogs in Korea as early as 2005 (Lee et al., 2012). Later, H3N2 CIV were discovered from sick dogs in China. The H3N2 CIV from China were phylogenetically similar to the Korean CIV isolates. The identified H3N2 CIV were of avian origin and no reassortment was observed (Li et al., 2010). H3N2 CIV isolates from Thailand also showed genetic relatedness to CIV subtype H3N2 of Korea and China (Bunpapong et al., 2014). Evolutionary studies revealed that the H3N2 CIV are the descendent of Eurasian and North American avian influenza viruses, which might have been prevailing unnoticed in dog population since the late of 1990s and early 2000s (Zhu et al., 2015).

Table 5: Summary of canine influenza outbreaks

Year	Country	investigation	Subtypes	Source	Severity	Reference
2002	UK	Outbreak	H3N8	Equine	No	(Daly et al., 2008)
2004	Thailand	Outbreak	H5N1	Avian	No	(Songserm et al., 2006)
2004	USA	Outbreak	H3N8	Equine	Yes	(Crawford et al., 2005)
2006	Canada	Surveillance	H3N8	Dog	No	(Kruth et al., 2008)
2007	S. Korea	Outbreak	H3N2	Avian	Yes	(Song et al., 2008)
2009	China	Experiment	H5N2	Dog	Yes	(Song et al., 2013)
2010	S. Korea	Surveillance	H3N1	Dog	No	(Song et al., 2012)
2010	China	Surveillance	H3N2	Dog	No	(Li et al., 2010)
2014	Taiwan	Surveillance	H6N1	Dog	Yes	(Lin et al., 2015)

A few years later, several reports of canine influenza virus emerged from other countries, such as Italy (Pratelli and Colao, 2014), Brazil (Mancini et al., 2012), and Canada (Kruth et al., 2008). In addition to H3N8 and H3N2, different subtypes of influenza A viruses, such as H1N1, H3N1,H3N2, H3N8, H5N1, H5N2 and H6N1 were also isolated from dogs (Kruth et al., 2008; Harder and Vahlenkamp, 2010; Zhang et al., 2013; Lin et al., 2015; Xie et al., 2016).

2.3.3 Canine influenza in Myanmar

In Myanmar, although HPAI H5N1 has been regularly reported in poultry, there is no report of influenza A virus infection in mammals, including dogs. Common illness with respiratory manifestations are frequently seen in dogs, particularly in stray dogs in villages in Myanmar. However, there is no report or confirmation of canine influenza in Myanmar. Lack of surveillance has been a major weakness in the epidemiology of canine influenza in Myanmar. To date, dog population in Myanmar has not been officially documented. There were approximately 3 million dogs in Myanmar in 2003. Current dog population in Myanmar was estimated to be around 4.5 million in 2015 (Data from YCDC)[3].

[3] Yangon City Development Committee, Unpublished data

2.4 Influenza A virus detection

2.4.1 Virus isolation

Isolation of the virus is an important assay for the diagnosis of influenza A virus infection. It is based on the isolation of virus in embryonated eggs or cell lines (Wozniak-Kosek et al., 2014). In avian species, samples for influenza virus isolation include oropharyngeal and cloacal swabs. In mammalian species, virus is often isolated from the nasal swab. For highly pathogenic avian influenza, the virus can be isolated from the blood and internal organs. Virus isolation is usually performed by inoculation of collected specimens into embryonated eggs or onto a tissue culture (Krauss et al., 2012).

2.4.2 Virus detection by real-time RT-PCR (rRT-PCR)

Detection of influenza A virus by real-time RT-PCR is a powerful diagnostic method for the screening of influenza A virus. Influenza A detection by real-time RT-PCR have advantages in terms of accuracy, convenience and rapidity. Real-time RT-PCR has high sensitivity and specificity, and therefore it can be used as a rapid diagnostic test (Pecoraro et al., 2013). It can differentiate mixed infections caused by different influenza subtype types (Wozniak-Kosek et al., 2014). Detection of avian and canine influenza virus can be readily achieved using real-time RT-PCR for screening and subtyping of the samples (Payungporn et al., 2008; Lu et al., 2010).

2.4.3 Serological assay for influenza virus detection

Serology is one of the useful diagnostic methods for detection of influenza A virus infection. When clinical specimens are unobtainable or when a laboratory resources are insufficient, serology diagnostic tools are good alternatives. Another advantage of serology tests is that genetic materials are not necessarily needed to be alive at the time of diagnosis. This is of a great value when the disease exposure is to be investigated in the absence of cold chain facilitation. There are different serological assays available for influenza A virus diagnosis, such as hemagglutination-inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA) and Agar Gel Immunodiffusion (AGID) test (Shafer et al., 1998; Zhang et al., 2013). Hemagglutination-inhibition (HI) assay is the test of choice for the serological detection of antibodies against influenza A virus infections in animals (Anderson et al., 2012). Hemmagglutination test is commonly used for routine serological detection (Hu et al., 2010; Zu et al., 2013; OIE, 2014). Alternatively, enzyme-linked immunosorbent assay (ELISA) can be used for the detection of antibodies for influenza A virus infection. ELISA is a rapid, sensitive, and specific diagnostic method for screening of large numbers of serum samples (de Boer et al., 1990; Brown et al., 2009).

CHAPTER III MATERIALS AND METHODS

This study was divided into 4 phases. The first phase was collection of samples from poultry and dogs in Myanmar. The second phase was isolation and identification of influenza A viruses. The third phase was serological survey of influenza A virus antibodies in poultry and dogs, and the fourth phase was genetic characterization of influenza A viruses isolated from Myanmar. Overview of materials and methods of this study is shown in figure 4.

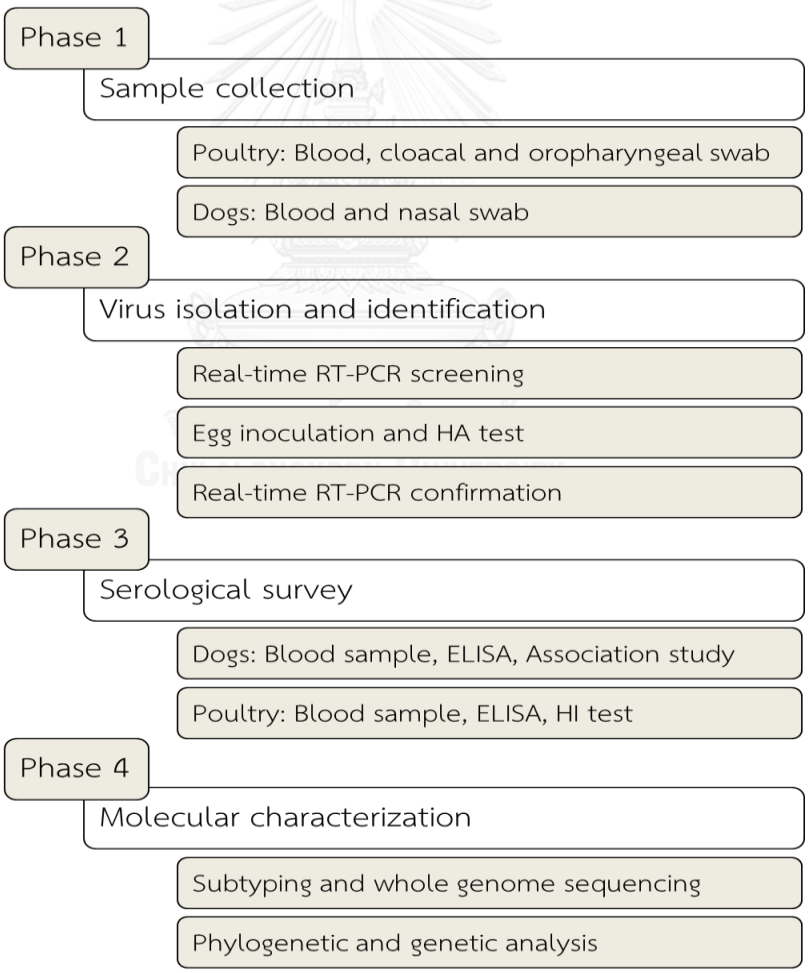

Figure 4: Flowchart of this study

3.1 Phase 1 Collection of samples from poultry and dogs in Myanmar

3.1.1 Sites of sample collection

In this study, three sampling areas, namely Namkham, Nay Pyi Taw and Yangon areas were selected as sampling sites. These areas were chosen as the sampling sites based on the criteria of high risk and transportation feasibility. Namkham locates in Muse administrative division in northeastern Shan state, border area to China. Nay Pyi Taw area is the central administrative region of Myanmar, composed of 8 townships. Yangon area is in the lower part of Myanmar and it has 33 townships (Figure 5).

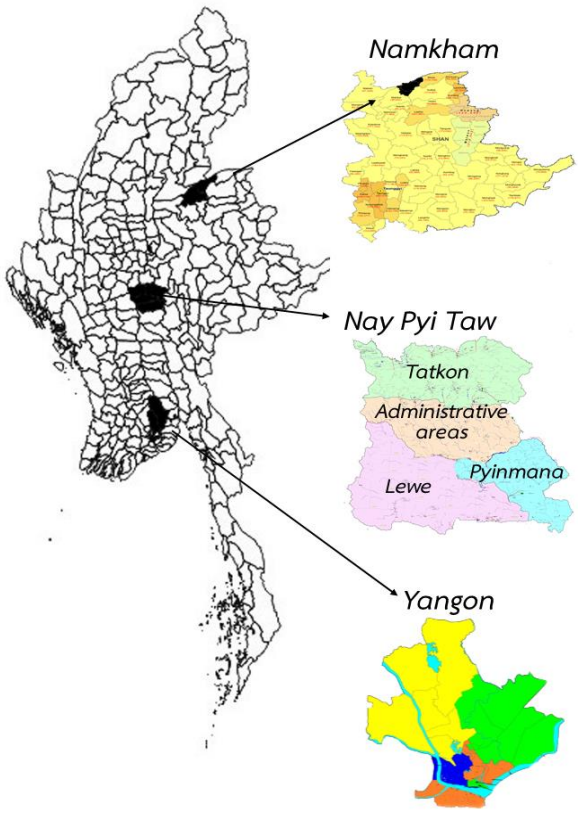

Figure 5: Namkham, Nay Pyi Taw, and Yangon areas of Myanmar

3.1.2 Live-bird markets for sample collection in poultry

Sample collection in poultry was carried out in live-bird markets (LBMs) in Namkham and Yangon (Figure 5 and 6). Two LBMs were selected for sample collection from poultry. The first LBM is in Mingalartaungnyun*t* township of Yangon known as Mingalartaungnyunt LBM. It is the biggest LBM in Myanmar, handling the average of 40,000 birds on daily basis. Chicken, ducks, and geese from peripheral townships of Yangon are transported and sold to this LBM. Customers, farmers and vendors from other townships visit this LBM for selling and buying live poultry and poultry products. Therefore, this LBM serves as the portal of entry for birds and provides ideal sampling site for avian influenza virus surveillance (Figure 7). The second market is in Namkham township. This LBM represents the poultry populations in border area between China and Myanmar. Namkham was considered a high-risk area for avian influenza infection according to previous serological surveillances and value chain study conducted by Livestock Breeding and Veterinary Department (LBVD), Myanmar[4]. In Namkham, delivery of poultry to LBM is different from Yangon LBM. Dealers collect poultry from several collecting points and sell at LBM. Sources of collection generally come from Ruili (Yunan province) of China. An average of 350 - 450 live poultry are killed and sold at Namkham LBM (Figure 8).

[4] Emergency surveillance & Emergency Response to AI H7N9 (Value Chain Study in China Border)

3.1.3 Dog shelters and villages for sample collection in dogs

For the surveillance of canine influenza virus in dogs in Myanmar, no notable risks exist among different locations since there was no previous reports or outbreaks of canine influenza virus infections in Myanmar. Therefore, sampling sites were focused on the areas with high numbers of dogs, particularly stray dogs. Sample collection in dogs was conducted in Yangon and Nay Pyi Taw areas. In Yangon, samples were collected from dog shelter located in Taukkyan township. In Nay Pyi Taw, sample collection was conducted in 14 villages in three townships, namely Tatkon, Pyinmana and Lewe. Among the eight townships of Nay Pyi Taw, the three townships included in this study are pre-existing townships and the other 5 are newly developed townships after the establishment of Nay Pyi Taw. The 5 new townships are merged as administrative areas, mainly occupied by governmental organizations (Figure 5).

3.1.4 Types of sample collection

Poultry samples

From June 2014 to August 2015, sample collection was carried out in poultry (Figure 6-8). Oropharyngeal and cloacal swabs from poultry, and environmental swabs from sites were collected using sterile rayon-tipped swab (Puritan®, Maine, USA). Animals were randomly selected to represent different sources, breeds and ages. Both healthy and sick animals with noticeable clinical signs such as sneezing,

nasal discharges, and swelling of eyelids, were targeted. In Yangon, samples were collected during three different seasons: rainy season (June and July 2014), winter (December 2014 and January 2015), and summer (April and May 2015). An average of 200 samples (100 oropharyngeal swab and 100 cloacl swabs) were collected each season. In Nankham, sample collection was conducted once a month from December 2014 to August 2015.

Dog samples

Nasal swab samples were collected from dogs during June 2014 through May 2015 (Figure 6). Sample collection was carried out in Yangon and Nay Pyi Taw. Swab samples were collected using sterile rayon-tipped swab (Puritan®, Maine, USA). Both healthy and clinically ill dogs apparently suffering from respiratory infection, such as sneezing, coughing, and dyspnea, were targeted. Dogs were randomly selected to represent different age, sex, and breed. In Yangon, samples were collected from dog shelter. In Nay Pyi Taw, sample collection was conducted in three townships, namely Pyinmana, Lewe, and Tatkon. Dogs from 14 villages in three townships were sampled (Table 11). Samples were collected during three different seasons: rainy season (June and July 2014), winter (December 2014 and January 2015), and summer (April and May 2015). Every season, at least 50 animals were sampled. Sampling sites for dog included households, monasteries, and clinics.

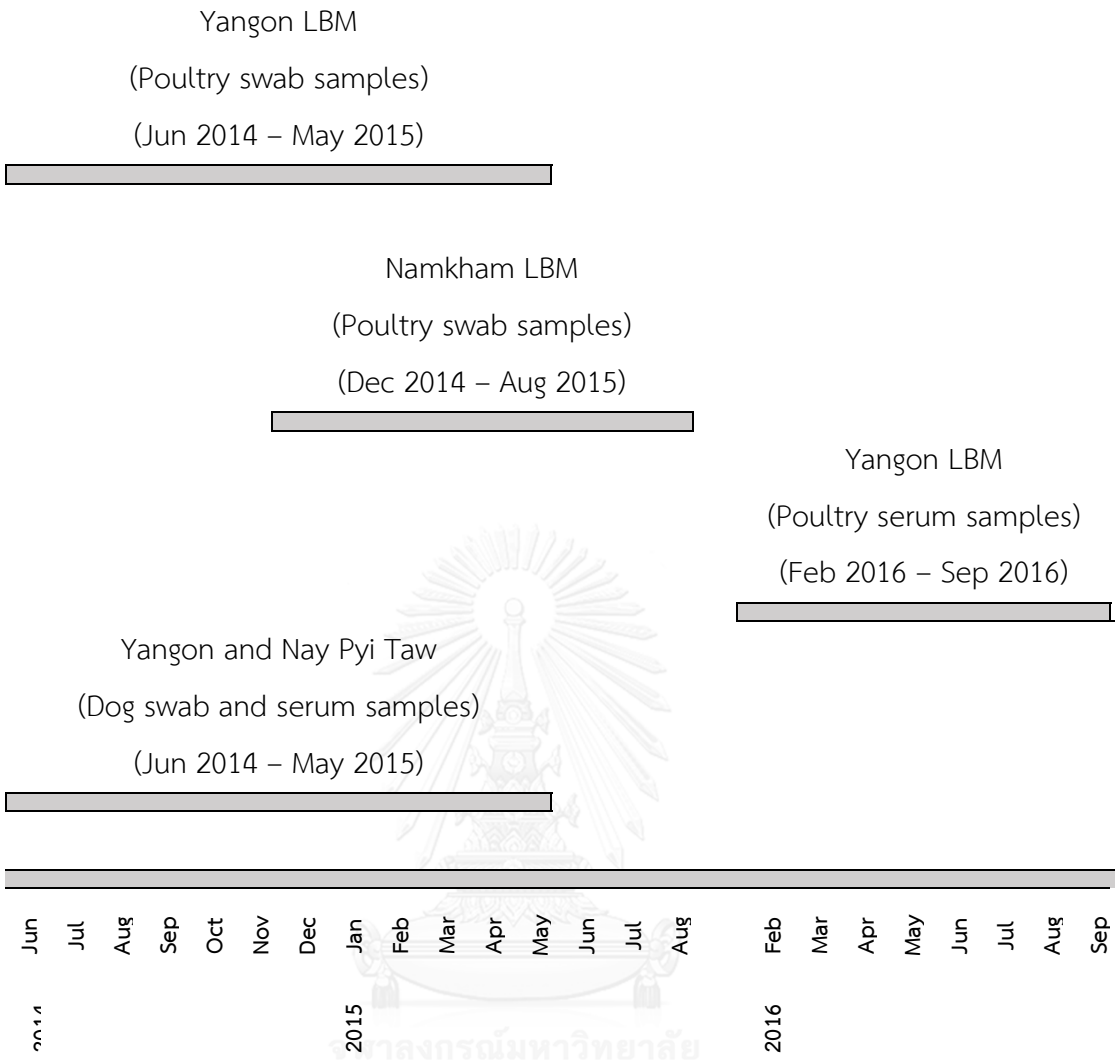

Figure 6: Timeline of sample collection

3.2 Phase 2 Isolation and identification of influenza A viruses

3.2.1 Sample preparation

Swab samples were placed into 2 ml tubes containing viral transport media (Brain-Heart Infusion broth supplemented with Penicillin G 1,000 U/ml, Streptomycin 1 mg/ml, Gentamycin 0.25 mg/ml and Kanamycin 0.5 mg/ml), and sent to laboratory within 24 hours after collection. At the laboratory, 1.5 ml of swab-embedded viral

transport media was allocated into 2 portions: 150 µl for RNA extraction and the rest for stock. Both of them were stored at -80°C until analysis.

3.2.2 RNA extraction and real-time reverse transcription polymerase chain reaction (rRT-PCR)

RNA extraction was performed by using NucleoSpin® RNA virus extraction kit (MACHEREY-NAGEL, GmbH & Co. KG, Germany) (APPENDIX A). The extracted RNA samples were then screened by real-time RT-PCR with Stratagene Mx3005P qPCR system (Agilent Stratagene, USA) to detect the Matrix (M) gene of influenza A viruses. Primers and probes were prepared as described in previous study (Spackman et al., 2002) (APPENDIX B). The results were interpreted by cycle threshold (Ct) value. The samples with Ct value greater than 40 were considered negative and the samples with Ct value less than 36 were considered positive. The samples with Ct value between 36 and 40 were considered as suspect. The suspected samples were inoculated for the second time and retested.

3.2.3 Influenza virus isolation

Real-time RT-PCR positive samples were then subjected to virus isolation by egg inoculation using 9 to 11 day-old embryonated chicken eggs, in accordance with OIE Terrestrial Manual 2014 (OIE, 2014). From each swab sample, 750 µl of supernatant was equally inoculated into three embryonated chicken eggs (250 µl per

egg). The Inoculated eggs were incubated at 37ºC for 72 hours and the death of embryo is examined every 12 hours. After 72 hours of incubation, the eggs were chilled at 4ºC overnight and allantoic fluid was collected from each egg. HA titers of the harvested fluid were determined by hemagglutination test (HA test) using 1% chicken RBC suspension. The samples that showed hemagglutination activity were considered HA positive. The allantoic fluid of HA positive samples showing HA titer ≥ 4 were kept at -80ºC for conformation by real-time RT-PCR. The samples were then tested for Matrix (M) gene of influenza A virus by real-time RT-PCR in the same ways as in the previous screening test (Spackman et al., 2002).

3.2.4 Influenza virus subtype identification

The Real-time positive RNA were then advanced to subtype identification by two-step reverse transcription PCR (RT-PCR) technique (Lee et al., 2001; Chen et al., 2007). First, RNA was reverse transcribed to cDNA using Improm-IITM Reverse Transcription System and random primer (Promega®, USA) (APPENDIX C). The synthesized cDNA was then used as the template for PCR amplification with specific primers (IAV HA 1-16 and NA 1-9 genes) for subtyping (Lee et al., 2001; Spackman et al., 2002; Tsukamoto et al., 2008; Tsukamoto et al., 2009) (APPENDIX D). The PCR products were then examined by gel electrophoresis using 1.2% of agarose gel in 0.5x Tris Borate EDTA (TBE). The agarose gel pad was scrutinized under the

ultraviolet (UV) light, and IAV subtypes were identified by analyzing the position of the bands corresponding to different IAV primer subtypes.

3.3 Phase 3 Serological survey for the presence of influenza A virus antibodies in poultry and dogs

3.3.1 Serological samples from poultry

A serological surveillance in poultry was conducted in Mingalartaungnyunt LBM, Yangon from February to September, 2016. An approximate of 3 ml of blood sample was collected from each animal. Poultry were randomly sampled to include different source, types and ages. Samples were collected once a month. At least, 60 animals were sampled in each time.

3.3.2 Serological samples from dogs

In dogs, serological surveillance was conducted from June 2014 to May 2015. An approximate of 5 ml of blood was collected from dogs. Dogs were randomly sampled regardless of breed, age and sex. Sample collection was carried out in one shelter from Yangon and villages in three townships in Nay Pyi Taw area (Table 11). Samples were collected during three different seasons: rainy season (June and July 2014), winter (December 2014 and January 2015), and summer (April and May 2015). Every season, at least 50 animals were sampled. Sampling sites for dog included households, monasteries, farms, and animal clinics.

3.3.3 ELISA test for influenza A antibodies in poultry

Collected blood samples were centrifuged at 3,000 rpm for 10 minutes. The sera were then kept at -20°C until tested. For poultry serum, blocking ELISA test kits (FlockCheck® AI Multi-Screen Ab Test kit, IDEXX Laboratories, USA) were used to detect the antibodies against the influenza A virus nucleoprotein (NP) (Brown et al., 2009). The ELISA test was performed following the manufacturer's instructions. First, the serum samples were diluted ten-fold by diluting 15 µl of serum with 135 µl of sample diluent. 100 µl of mixture, negative and positive control was dispensed into the avian influenza (AI) viral antigencoated plate and incubated at room temperature for 60 minutes. After washing with 350 µl of wash solution for 3 times, 100 µl of Anti-AI: Horseradish Peroxidase conjugate was added into each well and incubated at room temperature for 30 minutes. After washing, TMB substrate solution was added into each well and incubated at room temperature for 15 minutes. The reaction was stopped by adding 100 µl of stop solution. For result validation, the absorbance value of serum samples was examined by ELISA reader at A650 nm. The ELISA results were interpreted based on the test sample result-to-negative-control absorbance ratio (S/N%) value of each sample. The serum samples with a test sample result-to-negative-control absorbance ratio (S/N%) value greater than 0.7 (>70%) were considered negative for the presence of influenza A virus anti-nucleoprotein (NP) antibodies. The samples with test sample result-to-negative-control absorbance ratio (S/N%) value less than 0.6 (<60%) were considered positive.

The samples with (S/N%) values between 0.6 (60%) and 0.7 (70%) were considered as suspect.

3.3.4 ELISA test for influenza A antibodies in dogs

Blood samples from dogs were tested for the presence of antibodies against the influenza A virus nucleoprotein (NP) by using competitive ELISA test kits (ID Screen® Influenza A Antibody Competition MS Multi Species) (De Benedictis et al., 2010). The ELISA test was performed following the manufacturer's instructions. First, dog sera were diluted at 1:10 ratio with dilution buffer. Diluted sera were then incubated in 96-well plates coated with antigen A at 37°C for 1 hour. After incubation, each well was washed for 5 times with 300 µl of wash solution. Next, each well was filled with 50 µl of 1X conjugate and the plate was incubated at 25°C for 30 minutes. Following the incubation, each well was washed for 3 times with 300 µl of wash solution. Afterwards, 50 µl of substrate solution was added to each well and the plate was incubated at 25°C for 10 minutes in the dark. Finally, 50 µl of the stop solution was added to each well. The plate was read and optical density (OD) values were recorded at 450 nm. The ELISA results were interpreted based on the competition percentage of the samples. The competition percentage for each sample was calculated as here described: Competition % (S/N%) = (OD specimen / OD negative control) × 100. Serum samples with competition percentage less than 45% (0.45) were considered positive for the presence of influenza A virus anti-

nucleoprotein (NP) antibodies, and samples with competition percentage greater than 50% (0.50) were considered negative. Samples showing the competition percentage between 45% (0.45) and 50% (0.5) were taken as suspect.

3.3.5 Hemagglutination-inhibition (HI) assay

All avian serum samples were tested with hemagglutination-inhibition (HI) assay to identify specific hemagglutinin (HA) subtype. In this study, two subtypes of AIV (H5 and H9 of 4HAU) were used as the antigens. First, the serum samples were heated at 56ºC for 30 minutes, followed by 20% Kaolin treatment for 30 minutes at room temperature. After centrifugation at 3,000 rpm for 10 minutes, the Kaolin-treated sera were absorbed with 50% chicken red blood cells (RBC) and incubated at room temperature for 60 minutes to remove the non-specific inhibitor before HI test. The HI test was performed according to OIE guideline (OIE, 2016b). First, 25 µl of serum was diluted twofold by phosphate buffered saline (PBS) from 1:10 to 1:2560. Then, 25 µl of 4 HAU virus was mixed in each well and incubated the plate at room temperature for 60 minutes. After incubation, 50 µl of 1% chicken red blood cells was added into each well and the mixture was incubated at room temperature for 60 minutes. The antibody titer of the serum sample is measured by its ability to inhibit the agglutination at various dilutions. The HI titer is expressed as the reciprocal of the last dilution that completely inhibits the hemagglutination of the

RBC. The sera with HI titer less than 40 were considered negative. Sera showing HI titer greater than or equal to 40 were considered positive.

3.4 Phase 4 Genetic characterization of influenza A viruses

3.4.1 Whole genome sequencing

Whole genome sequencing was conducted in isolated influenza A viruses in this study. All eight gene segments were amplified using specific sets of oligonucleotide primer either available or newly designed by using Primer3 Input (Dakhave et al., 2013; Ren et al., 2013; Zhao et al., 2013). The amplified products were determined by agarose gel electrophoresis, and purified by using commercially available Nucleopin® PCR clean-up and gel extraction kits (Macherey -Nagel, Germany). Purified PCR products were then sent for nucleotide sequencing. The nucleotide sequences were assembled and validated using SeqMan software v.5.03 (DNASTAR Inc., Madison, WI, USA). Influenza A viruses available on the GenBank were used as references to compare with gene segments of the viruses in this study (Dong et al., 2011; Sharma et al., 2013; Parvin et al., 2014). The reference nucleotide sequences were included in the analysis to represent the avian influenza viruses from both Eurasian and North American lineages. Nucleotide sequences of each gene were aligned using Muscle within MEGA 6.0 (Tamura et al., 2013). Phylogenetic trees were constructed using the MEGA v.6.0 with neighbor-joining algorithm. Genetic

characteristics of the nucleotide sequences and deduced amino acids of each gene were analyzed using MEGA 6 (Hall, 2013; Tamura et al., 2013).

3.5 Statistics of this study

Microsoft office excel 2016 and statistic program for social science (SPSS for windows) version 20.0 (SPSS Inc., Chicago, USA) were used for statistical analysis in this study. Descriptive statistics was used to present the findings. Inferential statistics was applied to interpret the associations and correlations between hypothesized associated factors and disease status. Crosstab calculation with chi-square test was generated to find the association between the infection of influenza A viruses and hypothesized associated factors. Fisher's exact test was employed when the number of expected count in any cell of contingency table was less than 5. Confidence level was set at 95%. Associations that produced *p*-value less than 0.05 ($P<0.05$, two-tailed) were considered significant.

Figure 7: Mingalartaungnyunt live-bird market, Yangon

Figure 8: Namkham live-bird market

CHAPTER IV RESULTS

In this study, virological and serological surveillances for influenza A virus in poultry and dogs in Myanmar were conducted. Poultry and dog samples were collected from three sampling areas, namely Namkham, Nay Pyi Taw and Yangon areas. Namkham locates in Muse administrative division in northeastern Shan state, border area to China. Nay Pyi Taw area is in the central administrative region of Myanmar. Yangon area is in the lower part of Myanmar. In poultry, LBMs in Yangon and Namkham were surveyed. In dogs, shelters and villages from Yangon and Nay Pyi Taw areas were sampled. This influenza surveillance was conducted for 28 months, started from June 2014 and ended in September 2016.

For virological surveillance for influenza A virus in poultry, a total of 1,278 swab samples were collected. The swab samples were collected from ducks (n=380) and chicken (n=703). It was noted that, 630 swab (315 oropharyngeal and 315 clocal swab) samples were collected from LBM in Yangon, while 648 swab samples (453 oropharyngeal swabs and 195 environmental swabs) were collected from LBM in Namkham. For serological surveillance, the serum samples were collected from LBM in Yangon. A total of 621 blood samples were collected from ducks (n=132) and chickens (n=489) in the LBM (Table 6).

For virological surveillance for canine influenza in dogs, 203 nasal swabs were collected from dogs in 14 villages in Yangon and Nay Pyi Taw areas. A total of 31

dogs from Yangon and 172 dogs from Nay Pyi Taw were sampled. For serological surveillance, the serum samples were collected from 203 dogs (31 dogs from Yangon and 172 dogs from Nay Pyi Taw) (Table 6).

4.1 Occurrence of influenza A virus in poultry and dogs in Myanmar

4.1.1 Occurrence of Influenza A virus in poultry in LBMs in Myanmar

In this study, poultry in live-bird markets in Myanmar were found positive for influenza A virus by real-time RT-PCR screening test. The overall occurrence of influenza A virus infection in LBMs by real-time RT-PCR was 5.71% (73/1,278). In details, the occurrence of influenza A virus in LBMs was 7.62% (48/630) in Yangon LBM and 3.86% (25/648) in Namkham LBM. Among positive samples, 7.62% (24/315), 6.25% (48/768) and 0.51% (1/195) were found positive in cloacal swabs, oropharyngeal swabs and environmental swabs, respectively (Table 7). By months, influenza A occurrence in poultry was observed in December 2014- February 2015 and April – June 2015. The highest occurrence was observed in January 2015 (14.14%; 28/198). It was noted that influenza infection was highest (8.5%; 42/494) in winter (December – February). The occurrence was 2.1% (8/384) in rainy season (June – August) and 5.8%; (23/400) in summer (March – May) (Figure 9).

Table 6: Overall samples collected in this study

Place	Poultry				Dog	
	OS	CS	EV	Blood	NS	Blood
Namkham	453	-	195	-	-	-
Yangon	315	315	-	621	31	31
Nay Pyi Taw	-	-	-	-	172	172
Total	768	315	195	621	203	203

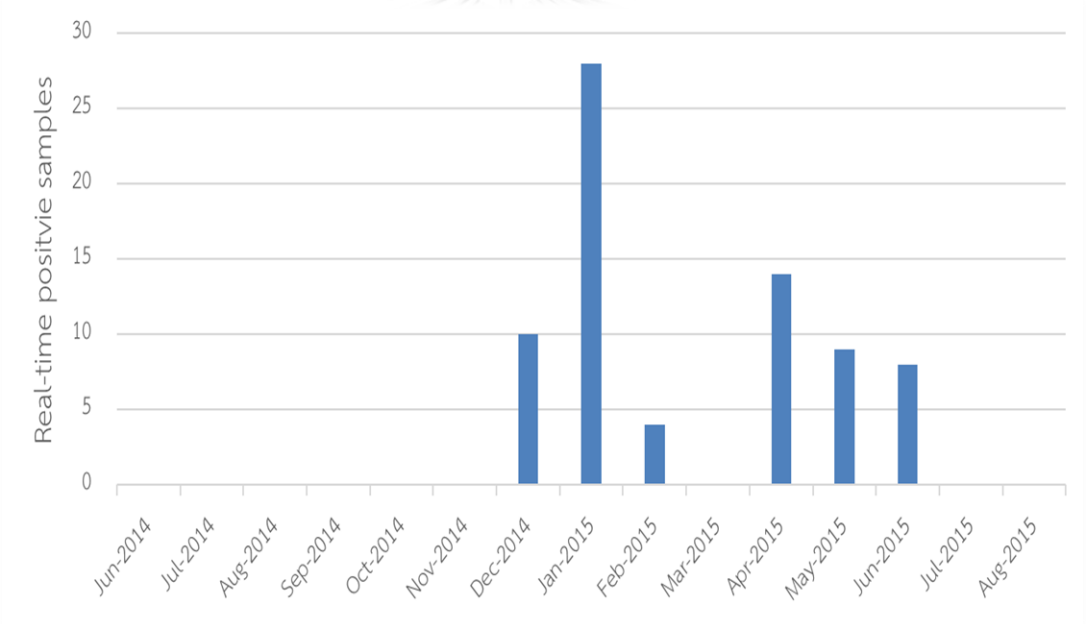

Figure 9: Occurrences of influenza A virus infection in poultry in live-bird markets during June 2014 to August 2015

Table 7: Number of real-time positive swab samples collected from poultry from Yangon and Namkham LBMs

Month	Yangon LBM						Namkham LBM					
	Chicken			Duck			Chicken		Duck		Environs	
	Birds	OS (%)	CS (%)	Birds	OS (%)	CS (%)	Birds	OS (%)	Birds	OS (%)	Swab	EV (%)
Jun 2014	41	0	0	9	0	0	-	-	-	-	-	-
Jul 2014	42	0	0	25	0	0	-	-	-	-	-	-
Dec 2014	30	2 (6.7)	4 (13.4)	18	1 (5.6)	2 (11.1)	42	1 (2.4)	28	0	30	0
Jan 2015	34	10 (29.4)	9 (26.5)	15	3 (20)	3 (20)	42	2 (4.8)	28	0	30	1 (3.4)
Feb 2015	-	-	-	-	-	-	42	4 (9.5)	28	0	30	0
Mar 2015	-	-	-	-	-	-	42	0	28	0	30	0
Apr 2015	31	2 (6.5)	1 (3.2)	20	6 (30)	5 (25)	21	0	12	0	15	0
May 2015	37	0	0	13	0	0	21	9 (42.8)	14	0	15	0
Jun 2015	-	-	-	-	-	-	21	8 (38.1)	14	0	15	0
Jul 2015	-	-	-	-	-	-	21	0	14	0	15	0
Aug 2015	-	-	-	-	-	-	21	0	14	0	15	0
Total	215	14 (6.5)	14 (6.5)	100	10 (10)	10 (10)	273	24 (8.8)	180	0	195	1 (0.51)

Table 8: Number of HA positive and virus isolation from poultry swab samples collected from Yangon and Namkham LBMs

Month	Yangon LBM										Namkham LBM								
	Chicken					Duck					Chicken			Duck			Environs		
		HA (%)		IAV (%)			HA (%)		IAV (%)			HA (%)	IAV (%)		HA (%)	IAV (%)		HA (%)	IAV (%)
	Bird	OS	CS	OS	CS	Bird	OS	CS	OS	CS	Bird	OS	OS	Bird	OS	OS	Swab	EV	EV
Jun 2014	41	0	0	0	0	9	0	0	0	0	-	-	-	-	-	-	-	-	-
Jul 2014	42	0	0	0	0	25	0	0	0	0	-	-	-	-	-	-	-	-	-
Dec 2014	30	0	0	0	0	18	0	0	0	0	42	1 (2.4)	0	28	0	0	30	0	0
Jan 2015	34	0	0	0	0	15	0	0	0	0	42	1 (2.4)	0	28	0	0	30	1 (3.4)	0
Feb 2015	-	-	-	-	-	-	-	-	-	-	42	0	0	28	0	0	30	0	0
Mar 2015	-	-	-	-	-	-	-	-	-	-	42	0	0	28	0	0	30	0	0
Apr 2015	31	0	0	0	0	20	0	0	0	0	21	0	0	12	0	0	15	0	0
May 2015	37	0	0	0	0	13	0	0	0	0	21	4 (19)	0	14	0	0	15	0	0
Jun 2015	-	-	-	-	-	-	-	-	-	-	21	3 (14.2)	3 (14.2)	14	0	0	15	0	0
Jul 2015	-	-	-	-	-	-	-	-	-	-	21	0	0	14	0	0	15	0	0
Aug 2015	-	-	-	-	-	-	-	-	-	-	21	0	0	14	0	0	15	0	0
Total	215	0	0	0	0	100	0	0	0	0	273	9 (3.3)	3 (1.1)	180	0	0	195	1	0

4.1.2 Distribution of influenza A virus in poultry

The distribution of IAV infection was 5.26% (20/380) in duck samples and 7.39% (52/703) in chicken samples. Among positive swab samples, 10 oropharyngeal (3.57%) and 10 cloacal (10.0%) swab samples were from ducks. In chicken, 7.78% (38/488) were from oropharyngeal swabs and 6.51% (14/215) were from cloacal swabs. Among different type of chicken, occurrence was highest (11.64%, 27/232) in broiler chickens. No virus was detected in backyard chickens (0/66). In some chicken flocks, different sub-species of chickens were mixed, and influenza A virus was detected in 8.79% (24/273) of mixed chickens (Table 9).

Table 9: Distribution of influenza A virus by species in poultry

	Test	RT-PCR (%)	Test	RT-PCR (%)	Test	RT-PCR (%)
Species	OS		CS		Total	
Duck	280	10 (3.57)	100	10 (10.0)	380	20 (5.26)
Chicken	488	38 (7.78)	215	14 (6.51)	703	52 (7.39)
Total	768	48 (6.25)	315	24 (7.62)	1083	72 (6.65)

Sub-species	OS		CS		Total	
Broiler chicken	116	14 (12.07)	116	13 (11.21)	232	27 (11.64)
Layer chicken	66	0	66	1 (1.52)	132	1(0.76)
Backyard chicken	33	0	33	0	66	0
Mixed chicken	273	24 (8.79)	0	0	273	24 (8.79)
Total	488	38 (7.79)	215	14 (6.51)	703	52 (7.4)

4.1.3 Occurrence of influenza A virus in dogs

In this study, 21.2% (43/203) of nasal swab samples were positive to influenza A virus by real-time RT-PCR. By location, the occurrence of influenza virus in dogs was 3.13% (1/31) in Yangon and 24.14 (42/172) in Nay Pyi Taw. The occurrence was highest (45.2%; 19/42) in December 2014. Among different seasons, occurrence was highest (43.03%; 34/79) in winter (December and January). For summer (April and May), no occurrence was detected in May. The occurrence was (8.3%; 6/72) in rainy season (Table 10).

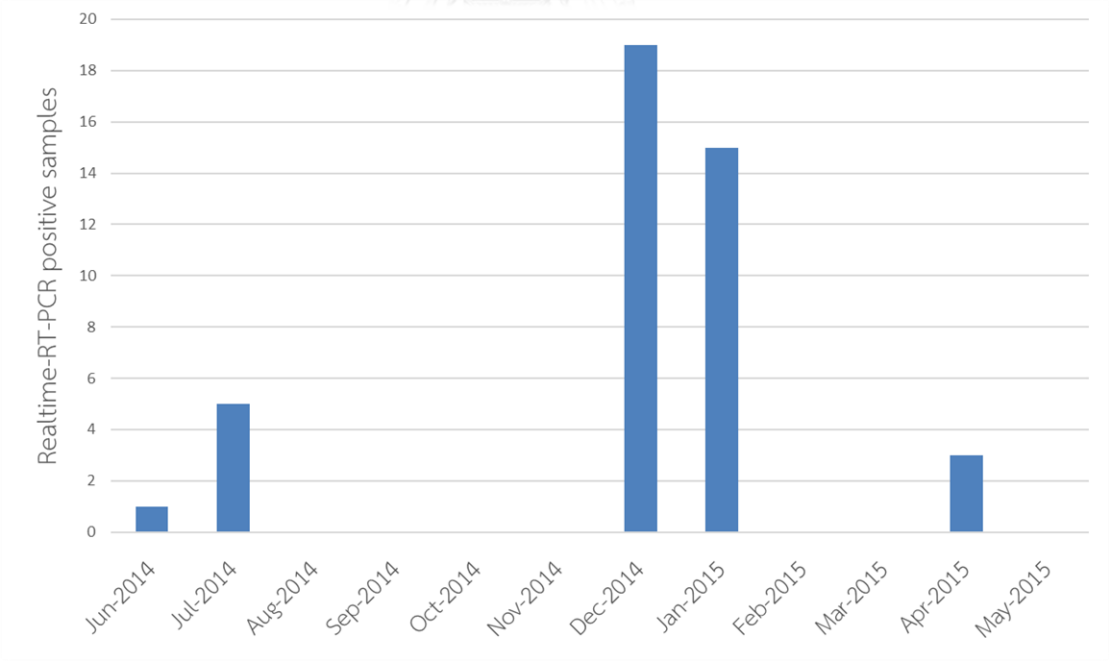

Figure 10: Occurrences of influenza A virus infection in dogs during June 2014 to May 2015

Table 10: Distribution of canine influenza virus in dogs tested by real-time-RT-PCR and virus isolation

Month	Yangon		Nay Pyi Taw						Total			
	Taukkyan		Pyinmana		Lewe		Tatkon					
	NS	RT-PCR (%)	NS	RT-PCR (%)	NS	RT-PCR (%)	NS	RT-PCR (%)	NS	RT-PCR (%)	HA	IAV
Jun 2014	31	1 (3.2)	0	0	-	-	-	-	31	1 (3.2)	0	0
Jul 2014	-	-	21	-	-	-	20	5 (25%)	41	5 (12.2)	0	0
Dec 2014	-	-	11	5 (45.5)	31	14 (45.2)	-	-	42	19 (45.2)	0	0
Jan 2015	-	-	14	5 (35.7)	9	4 (44.4)	14	6 (42.9)	37	15 (45.5)	0	0
Apr 2015	-	-	21	3 (14.3)	-	-	-	-	21	3 (14.3)	0	0
May 2015	-	-	-	-	14	0	17	0	31	0	0	0
Total	31	1 (3.2)	67	13 (19.4)	54	18 (33.3)	51	11 (21.6)	203	43 (21.2)	0	0

4.1.4 Distribution of influenza A virus in dogs

The occurrence of canine influenza in dogs was 3.2% (1/32), 33.3% (18/54), 19.4% (13/67), and 21.6% (11/51) in Taukkyan, Lewe, Pyinmana and Tatkon township, respectively. The occurrence of canine influenza in dogs was highest in Lewe township. Among villages, the occurrence of influenza A virus infection was observed in dog shelter in Taukkyan township and 9 villages in Lewe, Pyinmana, and Tatkon townships. The occurrence was highest (52.6%; 10/19) in Tha Pyay Kone village. No occurrence was observed in 5 villages (Table 11).

Table 11: Geographical distribution of canine influenza virus in dogs

Township	Village	Collection Points	Village level		Township level	
			NS	RT-PCR (%)	NS	RT-PCR (%)
Taukkyan	Dog shelter	S	31	1 (3.2)	31	1 (3.2)
Lewe	Tha Pyay Kone	H, M	19	10 (52.6)		
	Alar	H, M	12	4 (33.3)		
	Sama Taung	H	9	4 (44.4)		
	Shar Chaung	H, M	14	0		
	Total (lewe)				54	18 (33.3)
Pyinmana	Ma Oo Taw	H	10	0		
	Lay Thar	H, M	11	0		
	Sein Sar Pin	H	11	5 (45.5)		
	Yezin	H, C	14	5 (35.7)		
	Thar Yar Su	H	12	2 (16.6)		
	Kyaut Chat	H	9	1 (11.1)		
	Total (pyinmana)				67	13 (19.4)
Tatkon	New Yit	H	11	5 (45.5)		
	Tha Pyay Chaung	H	9	0		
	Zee Kone	H, F	14	6 (42.86)		
	Kin Thar	H, F	17	0		
	Total (Tatkon)				51	11 (21.6)
Total (all townships)					203	43 (21.2)

*H = Household, M = Monastery, F = Farm, C = Clinics, S = Shelter

4.1.5 Factors associated with canine influenza virus infection in dogs

General information pertaining to the dogs and dog owners were recorded, and the relationship between hypothesized associated factors and canine influenza status in dogs were analyzed. Answers from the owners were filled in sample collection sheets modified with general and management questions. Both modifiable management factors, such as the number of dogs living in the same household (yes, no), practice of vaccination (yes, no), and unmodifiable factors, such as age (< 2yrs, > 2 yrs), genders (female, male) were included. Chi-square test was used to analyze the relationship between canine influenza virus infection in dogs and hypothesized associated factors. Chi-square analysis showed that the age, gender, number of dogs in the household, confinement, and presence of poultry in the household did not produce significant association with CIV infection, while the presence of respiratory symptoms (p=0.004; OR=2.92; 95% CI=1.387 – 6.143), poor body condition score (p=0.015; OR=3.02; 95% CI=1.319 – 6.895), season of sampling (p=0.001; OR=8.31; 95% CI=3.224 - 21.426), and vaccination against influenza viruses (p=0.008; OR=9.69; 95% CI=1.283 - 73.24) showed statistically significant associations with canine influenza virus infections in dogs (Table 12).

Table 12: Association between canine influenza virus infections and hypothesized related factors in dogs

Category	RT-PCR Pos (%)	RT-PCR Neg (%)	Total	Odds ratio	*p*-value	95% CI
Age						
Older than 2 years	26 (19.4%)	108 (80.6%)	134	0.736	0.387	0.367 – 1.476
2 years and below	17 ((24.6%)	52 (75.4%)	69			
Gender						
female	23 (20.5%)	89 (79.5%)	112	0.917	0.802	0.467 – 1.803
male	20 (22.0%)	71 (78.0%)	91			
Keeping status						
Confined	6 (13.3%)	39 (86.7%)	45	0.503	0.144	0.198 – 1.282
Free	37 (23.4%)	121 (76.6%)	158			
Number of dogs						
Two and more	24 (19.8%)	97 (80.2%)	121	0.82	0.568	0.416 – 1.620
Only one	19 (23.2%)	63 (76.8%)	82			
Presence of poultry						
Yes	11 (28.2%)	28 (71.8%)	39	1.62	0.232	0.730 – 3.597
No	32 (19.5%)	132 (80.5%)	164			

* p-value is significant (<0.05) with chi-square test (2-tailed)

Category	RT-PCR Pos (%)	RT-PCR Neg (%)	Total	Odds ratio	*p*-value	95% CI
Respiratory symptoms						
Yes	16 (37.2%)	27 (62.8%)	43	2.92	0.004*	1.387 – 6.143
No	27 (16.9%)	133 (83.1%)	160			
Body condition score						
Good	6 (15.8%)	32 (84.2%)	38			
Medium	9 (12.5%)	63 (87.5%)	72			
poor	28 (30.1%)	65 (69.9%)	93	3.02	0.015*	1.319 – 6.895
Season						
Summer	3 (5.8%)	49 (94.2%)	52			
Rain	6 (8.3%)	66 (91.7%)	72			
Winter	34 (43.0%)	45 (5.70%)	79	8.31	0.001*	3.224 - 21.426
Vaccination						
Not vaccinated	42 (24.4%)	130 (75.6%)	172	9.69	0.008*	1.283 - 73.24
vaccinated	1 (3.2%)	30 (96.8%)	31			

* *p*-value is significant (<0.05) with chi-square test (2-tailed)

4.2. Influenza A virus Isolation and subtype identification

In this study, for influenza A virus isolation, 73 samples from poultry and 10 samples from dogs were influenza A positive by Real-time RT-PCR. All RT-PCR positive samples were then subjected to virus isolation by egg inoculation. No dog samples showed HA positive titer and 10 poultry samples showed HA titer. Out of 10 HA positive samples, 3 samples were confirmed positive for influenza A virus isolation (Table 13, Figure 11-13). For subtype identification, all three influenza viruses are identified as IAV subtype H9N2 viruses. All three viruses, designated as A/Chicken/Myanmar/NK-2/2015 (Sample ID: A 2815), A/Chicken/Myanmar/NK-4/2015 (Sample ID: A 2817), A/Chicken/Myanmar/NK-5/2015 (Sample ID: A 2818) were then subjected to genetic characterization (Table 13). In this study, the percentage of virus isolation was only 0.23% (3/1,278).

Table 13: HA test and rRT-PCR test results for virus isolation in this study

Sample ID	Date of collection	Location	Sample source	rRT-PCR Screening	HA titer	rRT-PCR confirmation	Ct value
A 2318	Dec-2014	Namkham	Chicken	positive	2^3	negative	negative
A 2429	Jan-2015	Namkham	Chicken	positive	2^3	negative	negative
A 2493	Jan-2015	Namkham	Environs	positive	2^3	negative	negative
A 2767	May-2015	Namkham	Chicken	positive	2^4	negative	negative
A 2768	May-2015	Namkham	Chicken	positive	2^4	negative	negative
A 2772	May-2015	Namkham	Chicken	positive	2^4	negative	negative
A 2773	May-2015	Namkham	Chicken	positive	2^4	negative	negative
A 2815	Jun-2015	Namkham	Chicken	positive	2^5	positive	8.178
A 2817	Jun-2015	Namkham	Chicken	positive	2^5	positive	9.469
A 2818	Jun-2015	Namkham	Chicken	positive	2^5	positive	13.585

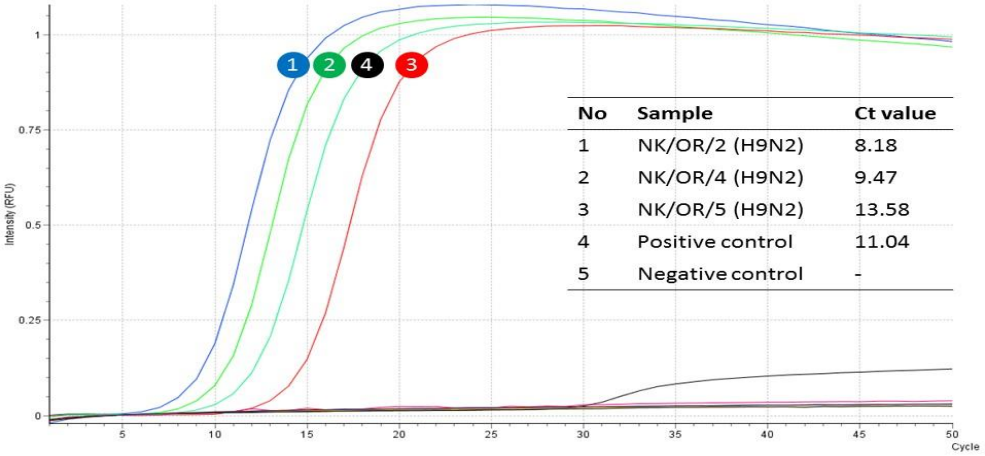

Figure 11: The result of rRT-PCR of three influenza A H9N2 subtype viruses isolated from this study. All three viruses were sequenced for whole genome.

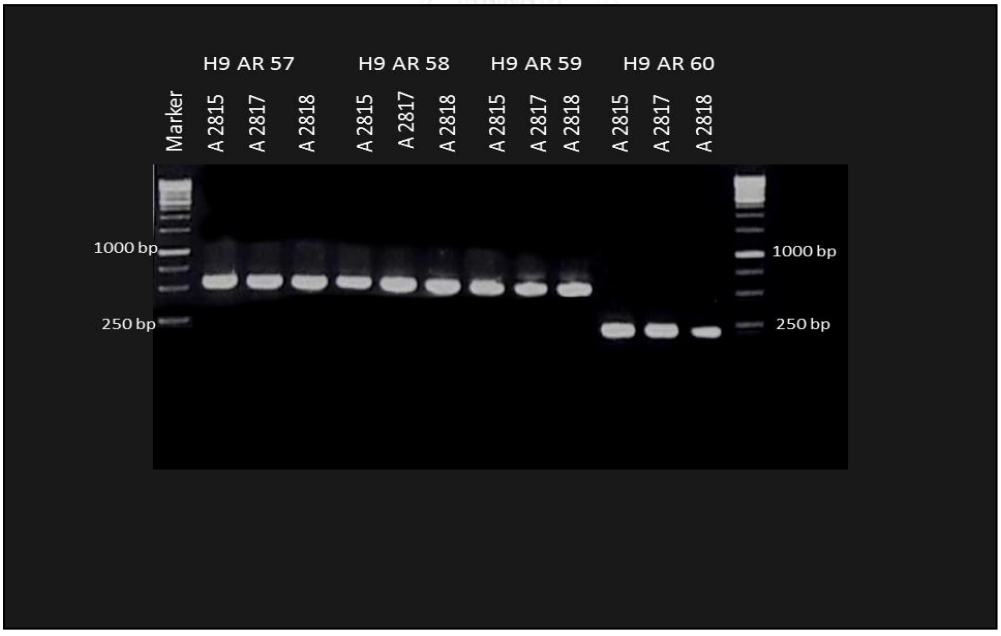

Figure 12: H9 subtype identification by PCR assay (1,000 bp marker) H9: expected PCR product: 610 bp (AR 57), 690 bp (AR 58), 570 bp (AR 59), 240 bp (AR 60)

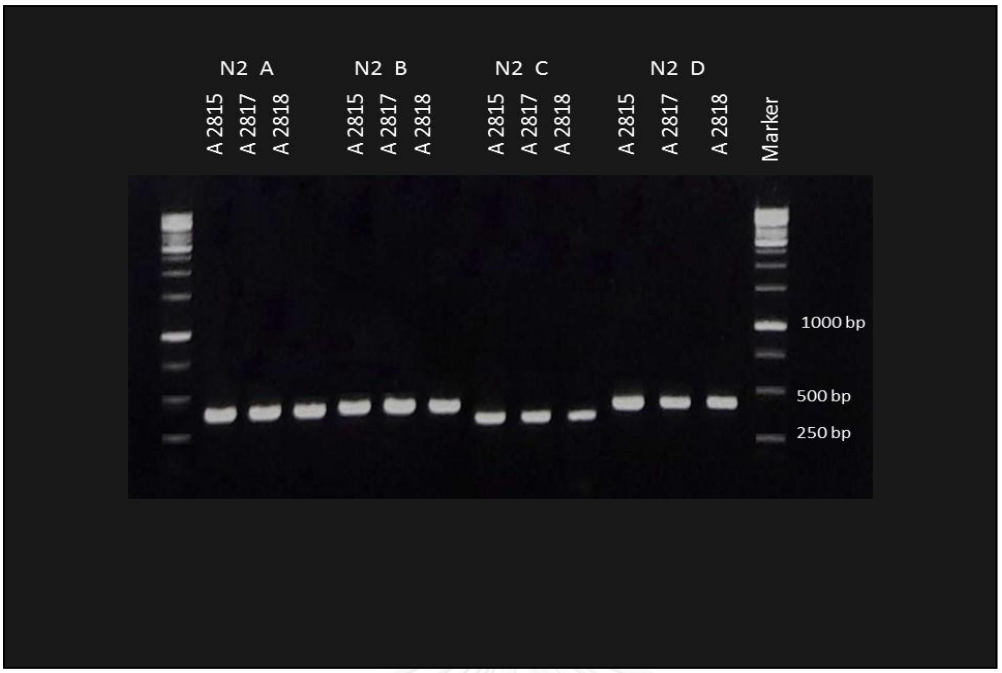

Figure 13: N2 subtype identification by PCR assay (1,000 bp marker) H9: expected PCR product: 460 bp (N2A), 460 bp (N2B), 390 bp (N2C), 460 bp (N2D)

4.3 Serological survey of influenza A virus antibodies in poultry and dogs

4.3.1 Occurrence of influenza antibodies in poultry by ELISA and HI tests

A total of 621 serum samples were collected from poultry during February 2016 through September 2016. Out of 621 sera, 12.8% (80 samples) was seropositive to influenza A by ELISA test. For HI test, 9 (1.44%) and 15 (2.4%) samples were seropositive to H5 and H9 influenza subtypes respectively (Table 14). It is noted that there were 18 (2.8%) samples that were positive to both ELISA and HI test (Table 15). In ELISA test, S/N% of positive samples ranged from, 4.69 to 43.39. For HI test, log values of positive samples ranged between 4 log 2 to 7 log 2 in H5 subtype, and 4 log 2 to 7 log 2 in H9 subtype.

In this study, percentages of seropositive by ELISA was highest in February (20.8%) and lowest in April (1.4%). For HI (H5) test, percentages of seropositive was highest in February (4.3%) and lowest in July (1.1%). No positive sample (H5 subtype) was detected in March, May, June and September. For H9 subtype, HI test results showed that the occurrence was higher in August (13.9%) and lower in June (5.4%) (Figure 14). No positive sample (H9 subtype) was detected in February, March, April, May, July and September.

Table 14: ELISA and HI results for serological survey of influenza A virus antibodies in poultry

Month	Tested	ELISA (+)	HI (H5)	HI (H9)
Feb 2016	91	19 (20.8%)	4 (4.3%)	0
Mar 2016	72	17 (23.6%)	0	0
Apr 2016	69	1 (1.4%)	1 (1.4%)	0
May 2016	68	10 (14%)	0	0
Jun 2016	74	15 (20.2%)	0	4 (5.4%)
Jul 2016	84	2 (2.3%)	1 (1.1%)	0
Aug 2016	79	16 (20.2%)	3 (3.7%)	11 (13.9%)
Sep 2016	84	0	0	0
Total	621	80 (12.8%)	9 (1.4%)	15 (2.4%)

Table 15: Description of ELISA and HI positive samples for serological survey of influenza A virus antibodies in poultry

Sample	Species	Detail	H5 Titer	H9 Titer	ELISA	S/N%
A 2374	Duck		4 log 2	negative	positive	15.64
A 2375	Duck		4 log 2	negative	positive	8.94
A 2376	Duck		5 log 2	negative	positive	36.69
A 2626	Chicken	Layer	negative	6 log 2	positive	5.46
A 2627	Chicken	Layer	negative	4 log 2	positive	8.14
A 2630	Chicken	Layer	negative	5 log 2	positive	5.08
A 2639	Chicken	Layer	negative	7 log 2	positive	4.69
A 2758	Chicken	Layer	4 log 2	7 log 2	positive	5.42
A 2759	Chicken	Layer	4 log 2	negative	positive	7.16
A 2760	Chicken	Layer	negative	4 log 2	positive	12.69
A 2762	Chicken	Layer	negative	5 log 2	positive	7.16
A 2763	Chicken	Layer	negative	4 log 2	positive	8.6
A 2765	Chicken	Layer	negative	4 log 2	positive	7.57
A 2766	Chicken	Layer	negative	4 log 2	positive	11.16
A 2767	Chicken	Layer	negative	6 log 2	positive	9.62
A 2769	Chicken	Layer	negative	4 log 2	positive	14.33
A 2770	Chicken	Layer	negative	7 log 2	positive	4.81
A 2771	Chicken	Layer	negative	4 log 2	positive	7.88

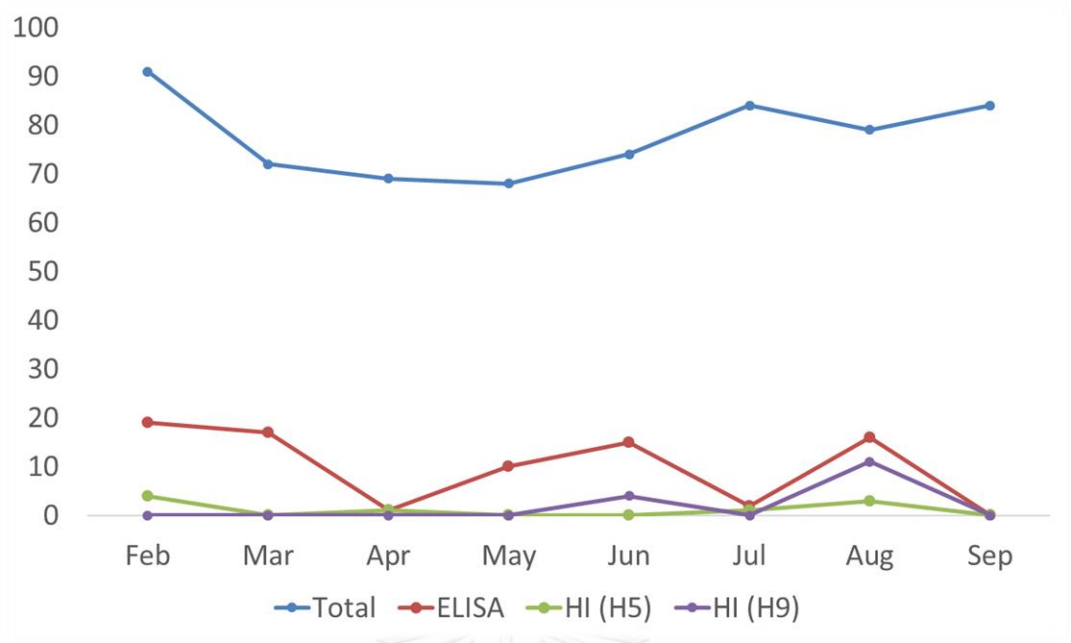

Figure 14: Occurrence of influenza A virus antibodies in poultry by month

4.3.2 Distribution of influenza A antibodies in poultry

A total of 132 duck and 489 chicken sera were tested. For ELISA, 13.0% (64/489) samples were seropositive in chicken and 12.1% (16/132) were seropositive in ducks. Among different types of chicken, percentages of seropositive was highest in layer chickens (38.1%; 51/134) by ELISA test. For HI (H5 subtype) test, percentages of seropositive was 3.7% (5/132) in ducks and 0.8% (4/489) in chickens. For HI (H9 subtype), no seropositive was detected in ducks and 3.0% (15/489) was seropositive in chickens. No HI (H9 subtype) seropositive sample was detected in broiler chickens (Table 16).

Table 16: Distribution of influenza A antibodies by species in poultry

Species	Tested	ELISA (%)	HI (H5) (%)	HI (H9) (%)
Duck	132	16 (12.1)	5 (3.7)	0
Chicken	489	64 (13.1)	4 (0.82)	15 (3.1)
Total	621	80 (6.25)	9 (1.5)	15 (2.4)

Sub-species	Tested	ELISA (%)	HI (H5) (%)	HI H9 (%)
Broiler chicken	242	5 (2.1)	1 (0.4)	0
Layer chicken	134	51 (38.1)	2 (1.5)	14 (10.4)
Backyard chicken	113	8 (7.1%)	1 (0.9)	1 (0.9)
Total	489	64 (13.1)	4 (0.82)	15 (3.1)

4.3.3 Occurrence of influenza antibodies in dogs by ELISA

In this study, 203 dog serum samples (31 from Yangon and 172 from Nay Pyi Taw) were tested. A total of 24 (11.8%) dog serum samples were found seropositive by ELISA detection. The S/N% of ELISA positive samples ranged from 17 to 43. By location, the occurrences of influenza antibodies in dogs were 11.6% (20/172) in Nay Pyi Taw and 12.9% (4/31) in Yangon. The occurrence was highest in January (29.7%) and lowest in July (7.3%). No occurrence was observed in April and May. As of the seasons, occurrence was highest (21.5%; 17/79) in winter (December and January). It was 9.7% (7/72) in rainy season (June and July) and there was no occurrence (0/52) in summer (April and May) (Table 17). In this study, six dogs that were positive to RT-PCR were also positive to ELISA detection (Table 18).

Table 17: ELISA result for serological survey of influenza virus antibodies in dogs

Month	Taukkyan		Pyinmana		Lewe		Tatkon		Total	
	Test	ELISA (%)		ELISA (%)	Test	ELISA (%)	Test	ELISA (%)	Test	ELISA (%)
Jun 2014	31	4 (13)	-	-	-	-	-	-	31	4 (12.9%)
Jul 2014	-	-	21	3 (14.3)	-	-	20	0	41	3 (7.3%)
Dec	-	-	11	3 (27.3)	31	3 (9.7)	-	-	42	6 (14.2%)
Jan 2015	-	-	14	7 (50%)	9	0	14	4 (28.6)	37	11 (29.7%)
Apr 2015	-	-	21		-		-		21	0
May	-	-	-		14		17		31	0
Total	31	1 (13)	67	13 (19.4)	54	3 (5.5)	51	4 (7.8)	203	24 (11.8%)

Table 18: Description of ELISA positive samples for serological survey of influenza

Dog ID	Township	Village	Month	CT value	S/N%
D 0096	Pyinmana	Sein Sar Pin	Dec 2014	16.27	29
D 0097	Pyinmana	Sein Sar Pin	Dec 2014	21.8	41
D 0122	Pyinmana	Yezin	Jan 2015	18.6	38
D 0128	Pyinmana	Yezin	Jan 2015	25.41	24
D 0129	Tatkon	Zee Kone	Jan 2015	5.53	30
D 0130	Tatkon	Zee Kone	Jan 2015	18.89	23

4.3.4. Distribution of serum antibodies in dogs

In Yangon, serum samples were collected from dog shelter in Taukkyan township. In Nay Pyi Taw, serum samples were collected from 14 villages in three townships. The occurrence of serum influenza antibodies was highest in Pyinmana township (19.4%; 13/67). It was 5.5% (3/54) in Lewe township, and 7.8% (4/51) in Tatkon township. Among villages, the occurrence of influenza A virus antibodies was observed in dog shelter and 5 villages. The occurrence was highest (50%; 7/14) in Yezin village. No occurrence was observed in 9 villages (Figure 15 and Table 19).

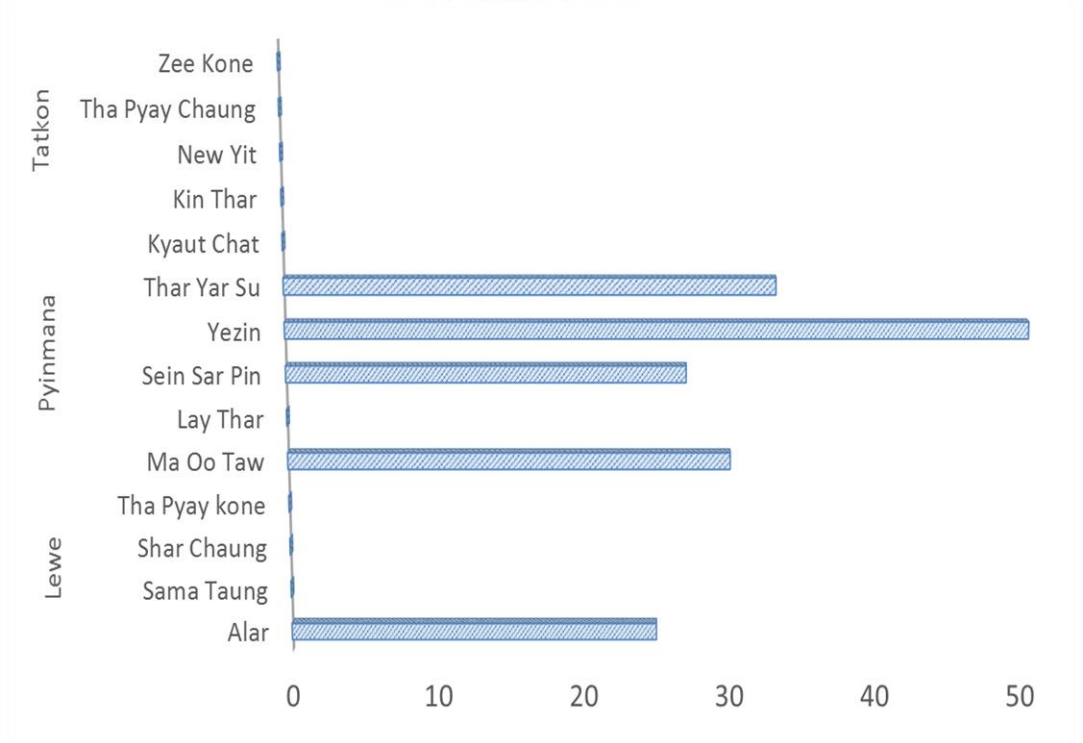

Figure 15: Distribution of influenza antibodies in dogs among villages

Table 19: Geographical distribution of influenza antibodies in dogs

Township	Village	Collection point	Village level		Township level	
			Blood	ELISA (%)	Blood	ELISA (%)
Taukkyan	Dog shelter	S	31	4 (13)	31	4 (13)
Lewe	Tha Pyay Kone	H, M	19	0		
	Alar	H, M	12	3 (25)		
	Sama Taung	H	9	0		
	Shar Chaung	H, M	14	0		
	Total (Lewe)				54	3 (5.5)
Pyinmana	Ma Oo Taw	H	10	3 (30)		
	Lay Thar	H, M	11	0		
	Sein Sar Pin	H	11	3 (27.3)		
	Yezin	H, C	14	7 (50)		
	Thar Yar Su	H	12	0		
	Kyaut Chat	H	9	0		
	Total (Pyinmana)				67	13 (19.4)
Tatkon	New Yit	H	11	0		
	Tha Pyay Chaung	H	9	0		
	Zee Kone	H, F	14	6 (28.6)		
	Kin Thar	H, F	17	0		
	Total (Tatkon)				51	4 (7.8)
Total (all townships)					203	24 (11.8)

*H = Household, M = Monastery, F = Farm, C = Clinics, S = Shelter

4.3.5 Factors associated with occurrence of influenza antibodies in dogs

General information pertaining to the dogs and dog owners were recorded, and the relationship between hypothesized associated factors and canine influenza status in dogs were analyzed. Answers from the owners were filled in sample collection sheets modified with some questions. Both modifiable management factors, such as the number of dogs living in the same household (yes, no), practice of vaccination (yes, no), and unmodifiable factors, such as age (< 2yrs, > 2 yrs), genders (female, male) were included. Chi-square test was used to analyze the relationship between the occurrence of serum influenza antibodies in dogs by ELISA and hypothesized associated factors. Chi-square analysis showed that the age, gender, number of dogs in the household, confinement, presence of poultry in the household, and vaccination against influenza did not produce any significant association with the occurrence of influenza antibodies, while the presence of respiratory symptoms (p=0.009; OR=3.16; 95% CI= 1.291 – 7.735) and season of sampling (p=0.001; OR=4.583; 95% CI= 1.804 – 11.645) showed significant associations with the occurrence of influenza antibodies in dogs (Table 20).

Table 20: Association between occurrence of influenza antibodies and hypothesized related factors in dogs

Category	ELISA Pos (%)	ELISA Neg (%)	Total	Odds ratio	*p*-value	95% CI
Age						
Older than 2 years	16 (11.9%)	118 (88.1%)	134	1.03	0.942	0.419 – 2.551
2 years and below	8 (11.6%)	61 (88.4%)	69			
Gender						
female	15 (13.4%)	97 (86.6%)	112	1.41	0.442	0.586 – 3.387
male	9 (9.9%)	82 (90.1%)	91			
Keeping status						
Confined	6 (13.3%)	39 (86.7%)	45	1.2	0.722	0.445 – 3.219
Free	18 (11.4%)	140 (88.6%)	158			
Number of dogs						
Two and more	14 (11.6%)	107 (88.4%)	121	0.94	0.892	0.397 – 2.237
Only one	10 (12.2%)	72 (8.78%)	82			
Presence of poultry						
Yes	5 (12.8%)	34 (87.2%)	39	1.12	0.787	0.391 – 3.218
No	19 (11.6%)	145 (88.4%)	164			

* *p*-value is significant (<0.05) with chi-square test (2-tailed)

Category	ELISA Pos (%)	ELISA Neg (%)	Total	Odds ratio	*p*-value	95% CI
Respiratory symptoms						
Yes	10 (23.3%)	33 (76.7%)	43	3.16	0.009*	1.291 – 7.735
No	14 (8.8%)	146 (91.2%)	160			
Body condition score						
Good	3 (7.9%)	35 (92.1%)	38			
Medium	6 (8.3%)	66 (91.7%)	72			
poor	15 (16.1%)	78 (83.9%)	93	2.158	0.243	0.897 – 5.191
Season						
Summer	0 (0%)	52 (100%)	52			
Rain	7 (8.5%)	65 (63.5%)	72			
Winter	17 (21.5%)	62 (78.5%)	79	4.583	0.001*	1.804 – 11.645
Vaccination						
Not vaccinated	20 (11.6%)	152 (88.4%)	172	0.888	0.768	0.282 – 2.802
vaccinated	4 (12.9%)	27 (87.1%)	31			

* *p*-value is significant (<0.05) with chi-square test (2-tailed)

4.3.6 Correlation between CIV status (RT-PCR and ELISA test positivity) and associated factors

Correlation between significantly associated factors and test results (RT-PCR and ELISA) were analyzed. Because both serum and swab were collected from same animals, the correlation between RT-PCR and ELISA results were also investigated. In this study, presence of respiratory symptoms and poor body condition scores were significantly correlated with both test (RT-PCR and ELISA) results. Seasons of sample collection and vaccination against influenza also showed significant correlation with the RT-PCR positives, but not with the ELISA positive samples (Table 21). However, no significant correlation (0.059, $p>0.05$) was observed between RT-PCR and ELISA test positive status.

Table 21: Correlation coefficient (r) between associated factors and CIV status (RT-PCR and ELISA)

Associated factors	RT-PCR (+)	ELISA (+)
Respiratory symptoms	0.215**	0.184**
Body conformation	0.212**	0.111
Collected season	0.414**	0.266**
Influenza vaccination	0.169**	- 0.014

** Correlation is significant at the 0.01 level (2 tailed)

4.4 Genetic characterization of influenza A viruses isolated from Myanmar

In this study, three IAV subtype H9N2 viruses isolated from chicken were characterized. It is to note that all three IAV subtype H9N2 isolates, namely A/Chicken/Myanmar/NK-2/2015(H9N2), A/Chicken/Myanmar/NK-4/2015(H9N2), and A/Chicken/Myanmar/NK-5/2015(H9N2) were collected from LBM in Namkham during June 2015. Whole genome sequencing was conducted (Table 22). The nucleotide sequences of all three IAV subtype H9N2 viruses were submitted to the GenBank. The details of the sequences and GenBank accession numbers for all three viruses are described in APPENDIX F.

Table 22: Description of influenza A virus subtype H9N2 characterized in this study

Sample ID	Subtype	Date collected	Location	Source	WGS
A/Chicken/Myanmar/ NK-2/2015	H9N2	Jun 2015	NK LBM	Chicken	Yes
A/Chicken/Myanmar/ NK-4/2015	H9N2	Jun 2015	NK LBM	Chicken	Yes
A/Chicken/Myanmar/ NK-5/2015	H9N2	Jun 2015	NK LBM	Chicken	Yes

4.4.1 Nucleotide similarities of Influenza A viruses subtype H9N2 comparing to the Gene Bank database (BLAST analysis)

The nucleotide sequences of all three IAV subtype H9N2 isolates from this study were compared with reference sequences of other IAV isolates in GenBank, using the NCBI nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST analysis showed that all three IAV subtype H9N2 isolates from this study were closely related to IAV subtype H9N2 isolates from chicken and environmental samples from China (Table 23). Percentages of nucleotide similarity was (99%) among the isolates.

Table 23: Nucleotide identities between IAV subtype H9N2 isolates in this study and reference sequences of other influenza A viruses in Genbank

Gene	Position	Genbank accession number	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	1-2280	KT356726.1	A/environment/Hunan/27420/2014 (H9N2)	99%
PB1	1-2274	KT356735.1	A/environment/Hunan/25998/2014 (H9N2)	99%
PA	1-2151	KP414330.1	A/chicken/Shenzhen/1047/2013 (H9N2)	99%
HA	1-1683	KP766779.1	A/chicken/Guangdong/G3532/2014 (H9)	99%
NP	1-1497	KP416465.1	A/chicken/Dongguan/1693/2014(mixed)	99%
NA	1-1401	KP414447.1	A/chicken/Shenzhen/1949/2013(H9N2)	99%
M	1-968	KT356784.1	A/environment/Hunan/18355/2014(H9N2)	99%
NS	1-852	KT699060.1	A/Anser fabalis/Anhui/L139/2014(H9N2)	99%

4.4.2 Phylogenetic analysis of influenza A virus subtype H9N2 in Myanmar

Phylogenetic analysis of HA gene showed that all three IAV subtype H9N2 viruses were grouped into the H9.4.2.5 sub-lineages. The H9.4.2.5 sub-lineage belongs to H9.4.2 lineages represented by reference IAV-H9N2, Y280 or BJ94 (Figure 16). For phylogenetic analysis of NA gene, all three IAV subtype H9N2 viruses of this study were clustered into BJ-94 lineage (Figure 17). Phylogenetic analysis of internal genes also provided similar results that Myanmar H9N2 viruses were closely related to IAV subtype H9N2 viruses recovered in China during 2013 and 2015 (Figure 18 – 23).

4.4.3 Genetic analysis of influenza A virus subtype H9N2 in Myanmar
HA and NA genes

For genetic analysis, the deduced amino acids of three IAV-H9N2 viruses were compared with other H9N2 viruses in the GenBank using MegAlign software (DNASTAR). The amino acids at HA cleavage site, receptor-binding residues, left edge of receptor binding pockets and right edge of receptor binding pockets were analyzed. The genetic analysis of HA genes revealed that all three isolates had R-S-S-R connecting peptide at the HA cleavage site, which is common in land-based avian influenza viruses (Webster et al., 1992), and represent low pathogenicity in chickens (Munir et al., 2013; Stech and Mettenleiter, 2013). The presence of 226L motif at the receptor binding sites of HA gene suggested that these viruses have

preferential affinity for human-like α-2,6-linked sialic acid receptors (Matrosovich et al., 2001). For NA gene analysis, all three isolates had a 3-amino acid deletion (positions 62 to 64) in NA stalk region, which is necessary for adaptation of the virus from wild birds to poultry (Matrosovich et al., 1999) and increased virulence of the influenza viruses (Stech et al., 2015) (Table 24).

Internal genes

In PB1 genes, host specific avian-human amino acid was observed at 13P (Wernery et al., 2013). No mutation was observed at amino acid position 701 and 627 in PB2 proteins, retaining the avian characteristics (Yuan et al., 2015). On M2 protein of all three isolates carried S31N substitution, indicating the resistance to amantadine (Aamir et al., 2007). The PA protein carried 409N substitution associated with mammalian adaptation and 672L indicated increased virulence (Kandeil et al., 2014). V149A was found on NS1 protein, which may antagonize interferon induction in chicken fibroblast (Li et al., 2004) (Table 25).

Table 24: Genetic analysis of HA and NA genes of influenza A viruses subtype H9N2 from Myanmar

	HA binding site (H3 Numbering)								NA stalk deletion		
	158	183	189	190	226	227	228	Cleavage site	38-39	46-50	62-64
A/Chicken/Beijing/1/1994	N	N	T	V	Q	Q	G	PARSSR/G	No	No	No
A/Duck/Hong Kong/Y280/1997	N	N	T	T	L	Q	G	PARSSR/G	No	No	Yes
A/Quail/Hong Kong/G1/1997	S	H	T	E	L	Q	G	PARSSR/G	Yes	No	No
A/Duck/Hong Kong/Y439/1997	S	H	T	E	Q	Q	G	PAASNR/G	No	No	No
A/Chicken/Shanghai/F/1998	N	N	T	A	Q	Q	G	PARSSR/G	No	No	Yes
A/Chicken/Fujian/C1161/2013	N	N	T	A	L	Q	G	PARSSR/G	No	No	Yes
A/Chicken/Shanghai/06/2015	N	N	D	T	L	M	G	PSRSSR/G	No	No	Yes
A/Chicken/Myanmar/NK-2/2015	N	N	T	T	L	M	G	PSRSSR/G	No	No	Yes
A/Chicken/Myanmar/NK-4/2015	N	N	T	T	L	M	G	PSRSSR/G	No	No	Yes
A/Chicken/Myanmar/NK-5/2015	N	N	T	T	L	M	G	PSRSSR/G	No	No	Yes

Table 25: Genetic analysis of internal genes of influenza A viruses subtype H9N2 from Myanmar

Virus	PB2			PB1	PA			M1	M2				NS1		
	27	627	701	13	356	409	672	15	27	28	31	55	42	149	217
A/Chicken/Beijing/1/1994	H	E	D	P	K	S	L	I	V	V	S	F	S	A	K
A/Duck/Hong Kong/Y280/1997	H	E	D	P	K	S	L	I	V	V	S	F	S	A	K
A/Quail/Hong Kong/G1/1997	H	E	D	P	K	S	L	I	V	V	S	F	S	A	K
A/Duck/Hong Kong/Y439/1997	H	E	D	P	K	S	L	V	V	I	S	L	S	A	K
A/Chicken/Shanghai/F/1998	H	E	D	P	K	N	L	I	V	V	N	F	S	A	K
A/Chicken/Fujian/C1161/2013	H	E	D	P	K	S	L	I	V	V	N	F	S	A	K
A/Chicken/Shanghai/06/2015	H	E	D	P	R	N	L	I	V	V	N	F	S	A	K
A/Fujian/1/2013	H	K	D	P	R	N	L	I	V	V	N	F	S	A	K
A/Shanghai/01/2014	H	K	D	P	R	N	L	I	V	V	N	F	S	A	K
A/Chicken/Myanmar/NK-2/2015	H	E	D	P	R	N	L	I	V	V	N	F	S	A	K
A/Chicken/Myanmar/NK-4/2015	H	E	D	P	R	N	L	I	V	V	N	F	S	A	K
A/Chicken/Myanmar/NK-5/2015	H	E	D	P	R	N	L	I	V	V	N	F	S	A	K

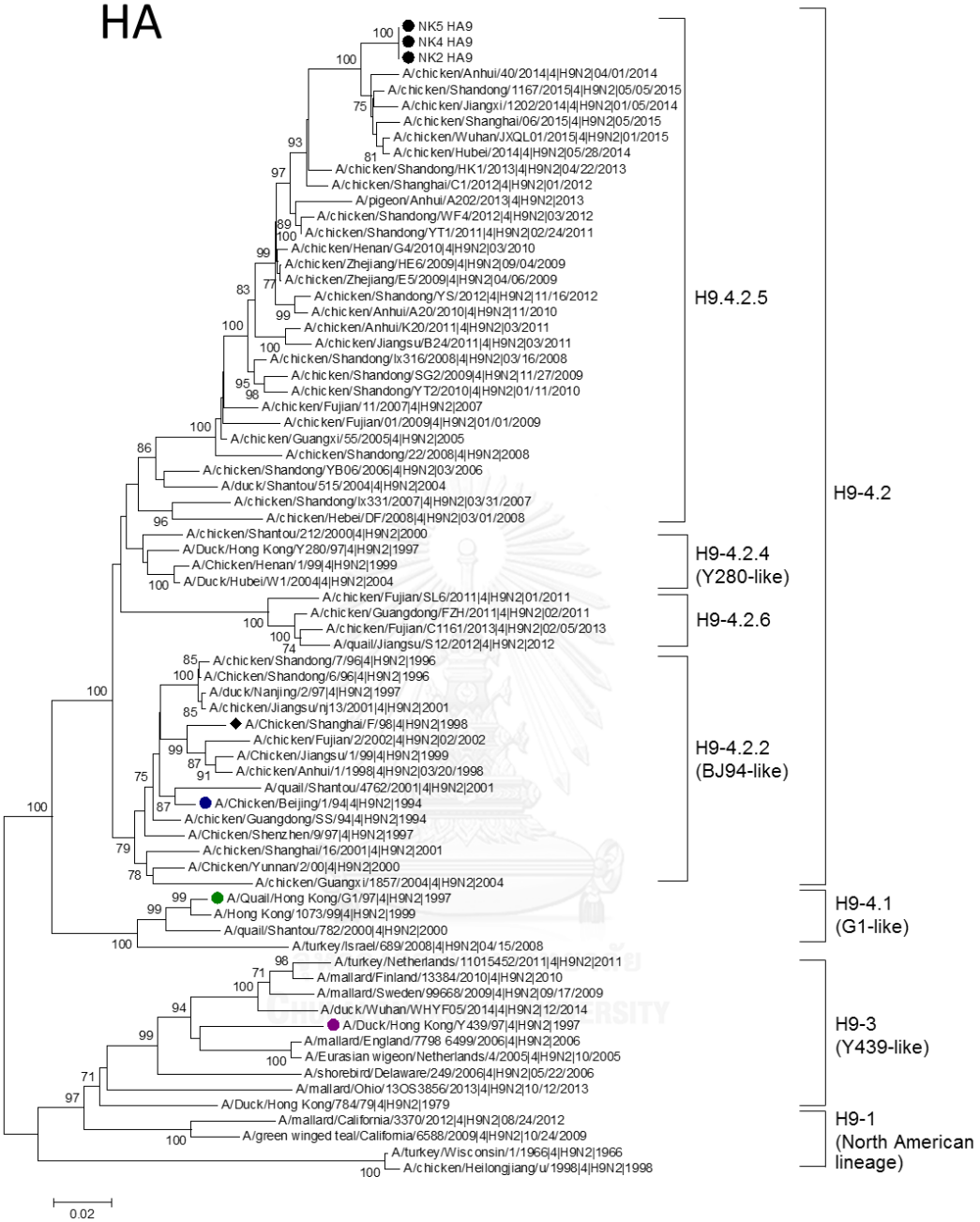

Figure 16: Phylogenetic analysis of the HA9 genes of all three H9N2 isolates

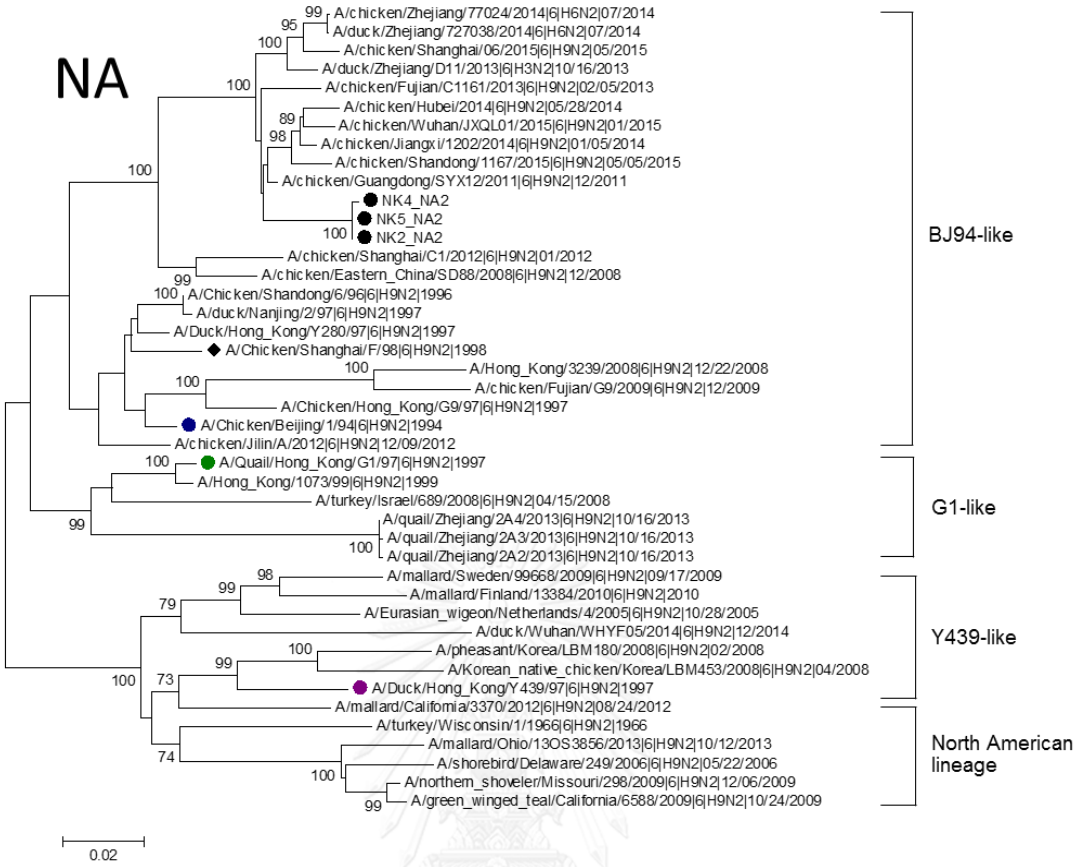

Figure 17: Phylogenetic analysis of the NA2 genes of all three H9N2 isolates

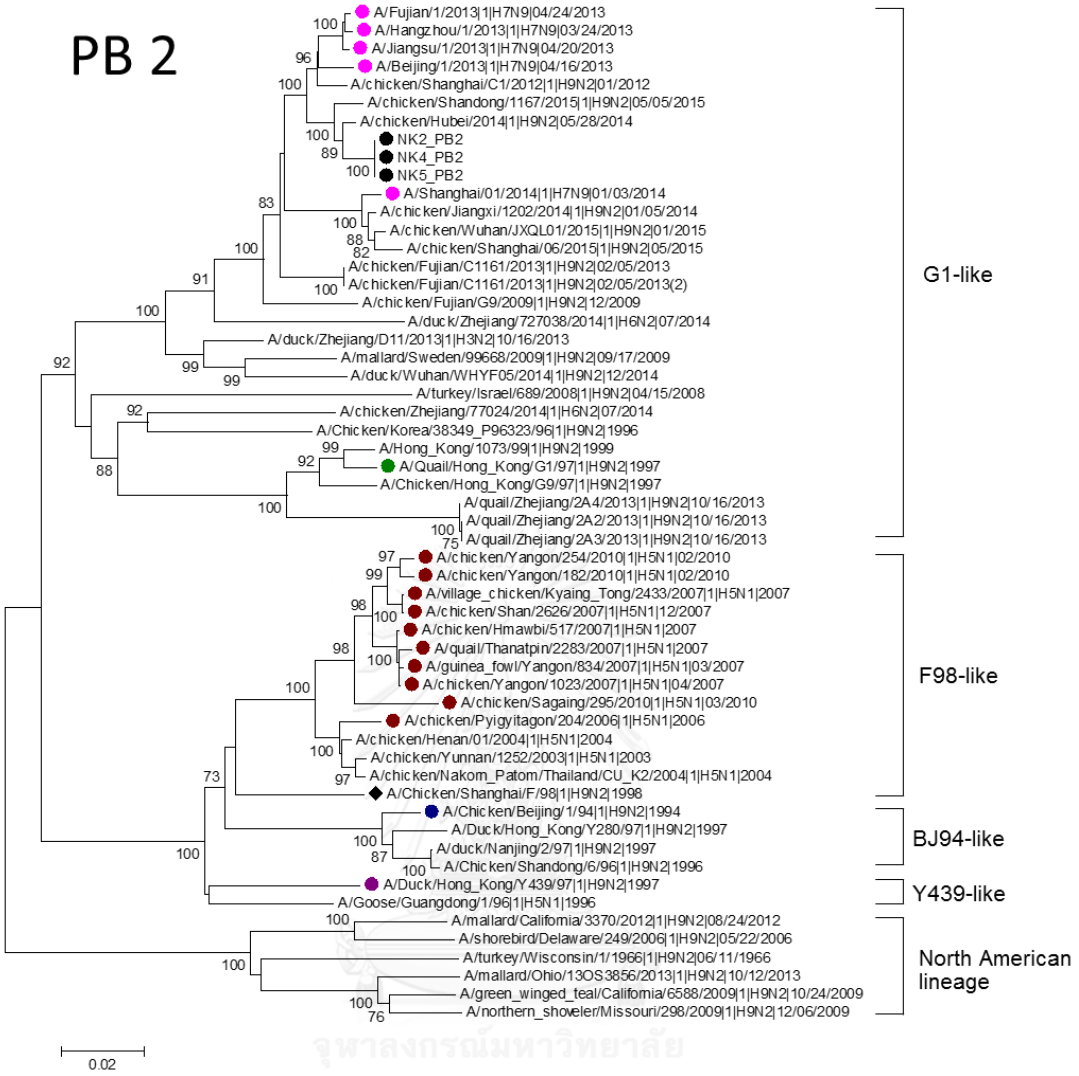

Figure 18: Phylogenetic analysis of the PB2 genes of all three H9N2 isolates

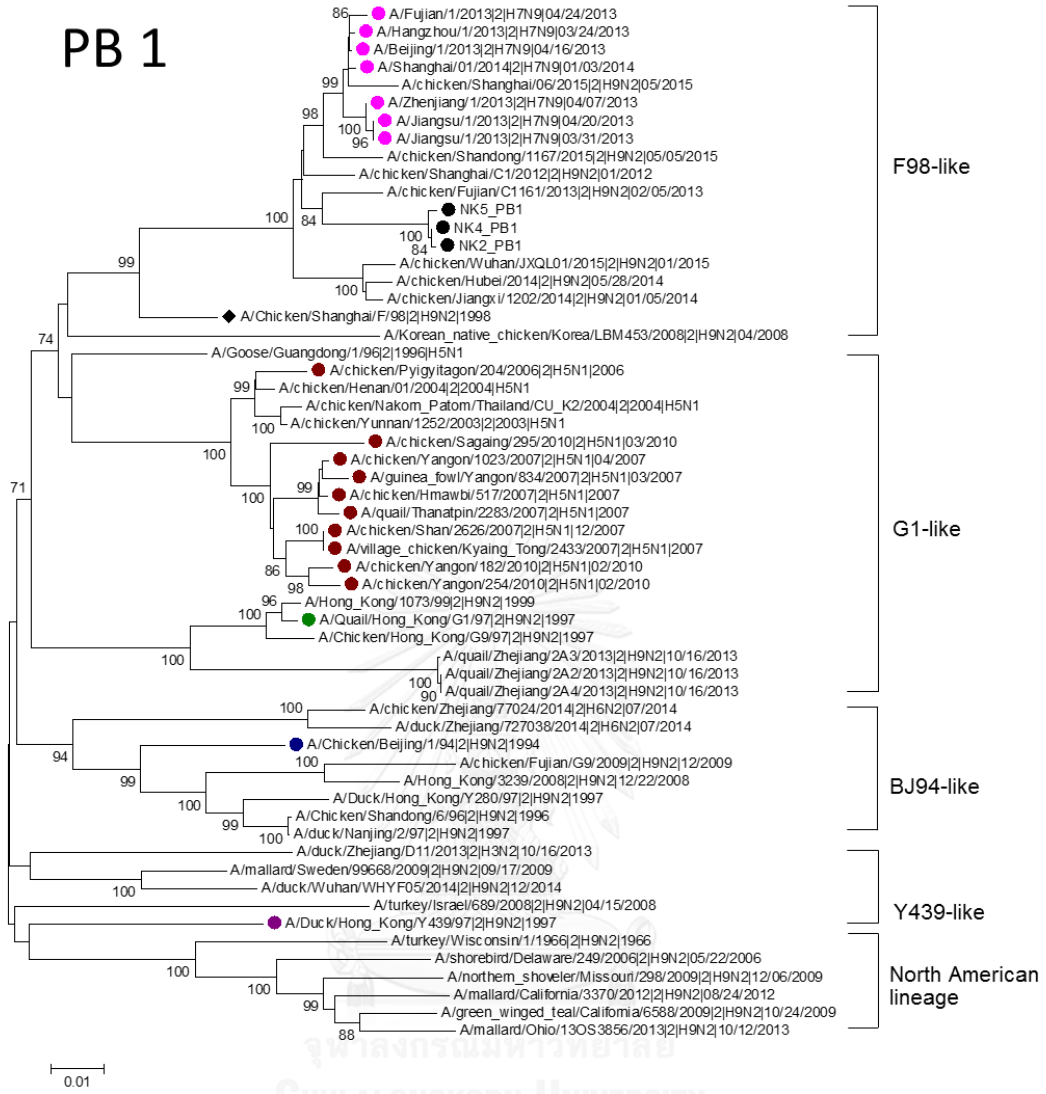

Figure 19: Phylogenetic analysis of the PB1 genes of all three H9N2 isolates

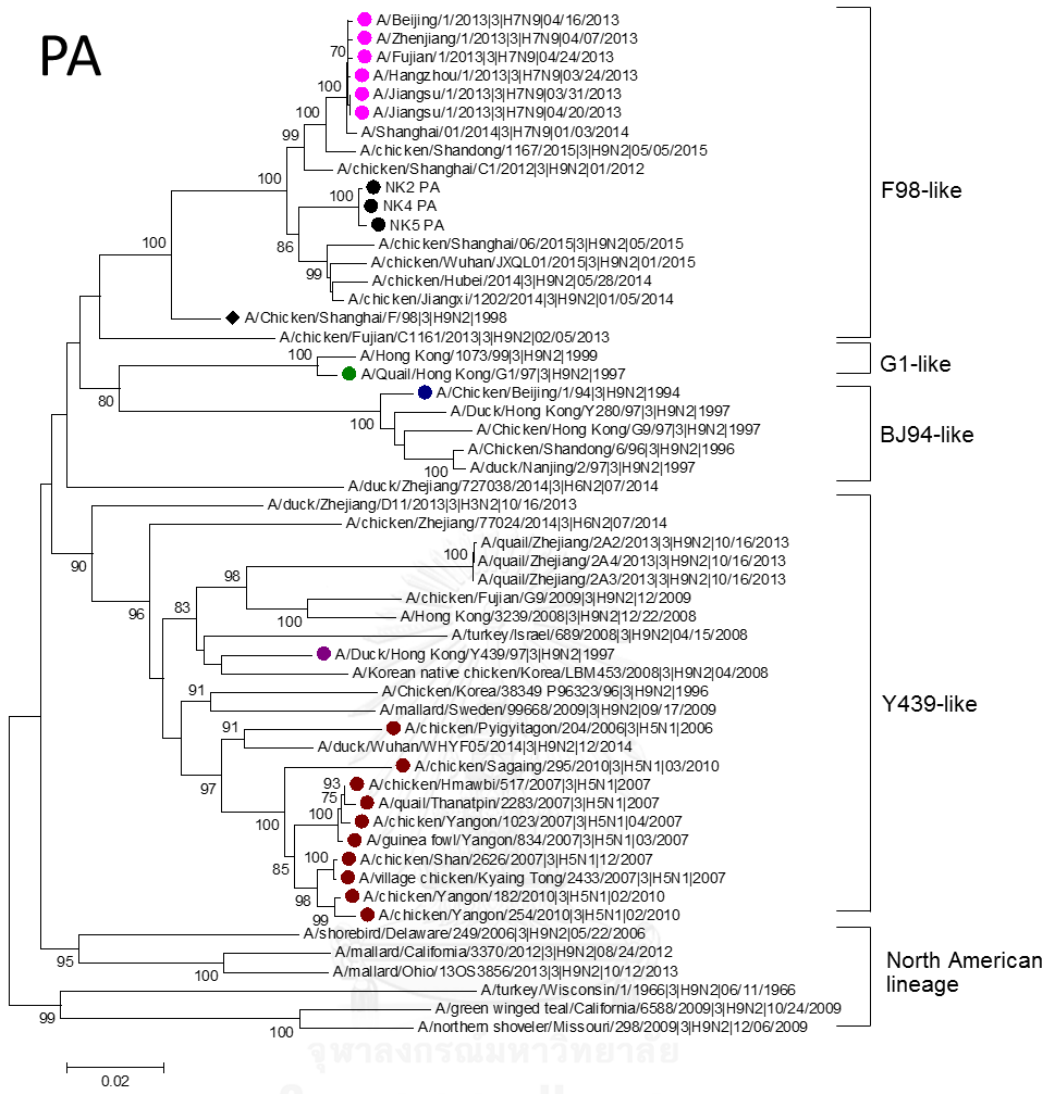

Figure 20: Phylogenetic analysis of the PA genes of all three H9N2 isolates

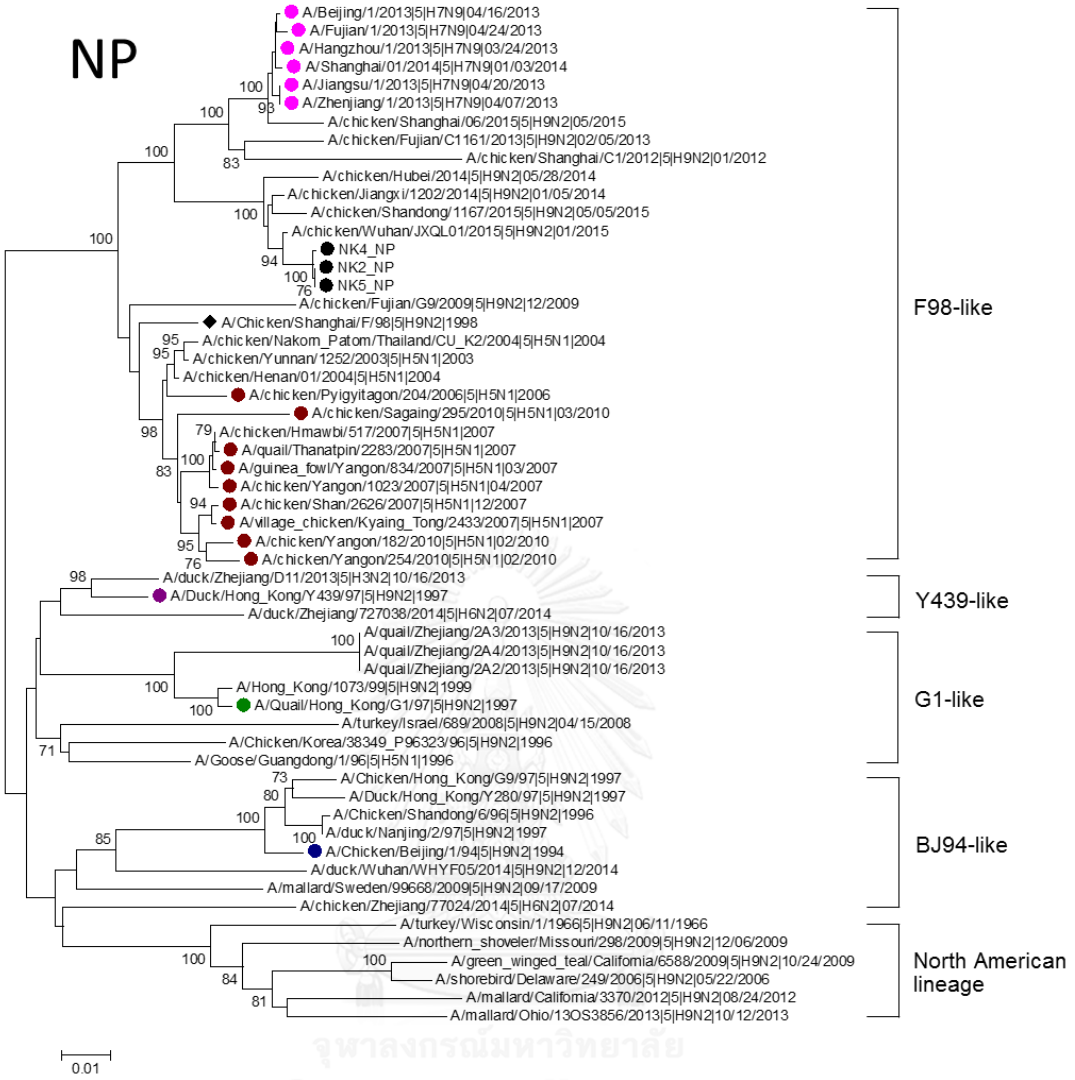

Figure 21: Phylogenetic analysis of the NP genes of all three H9N2 isolates

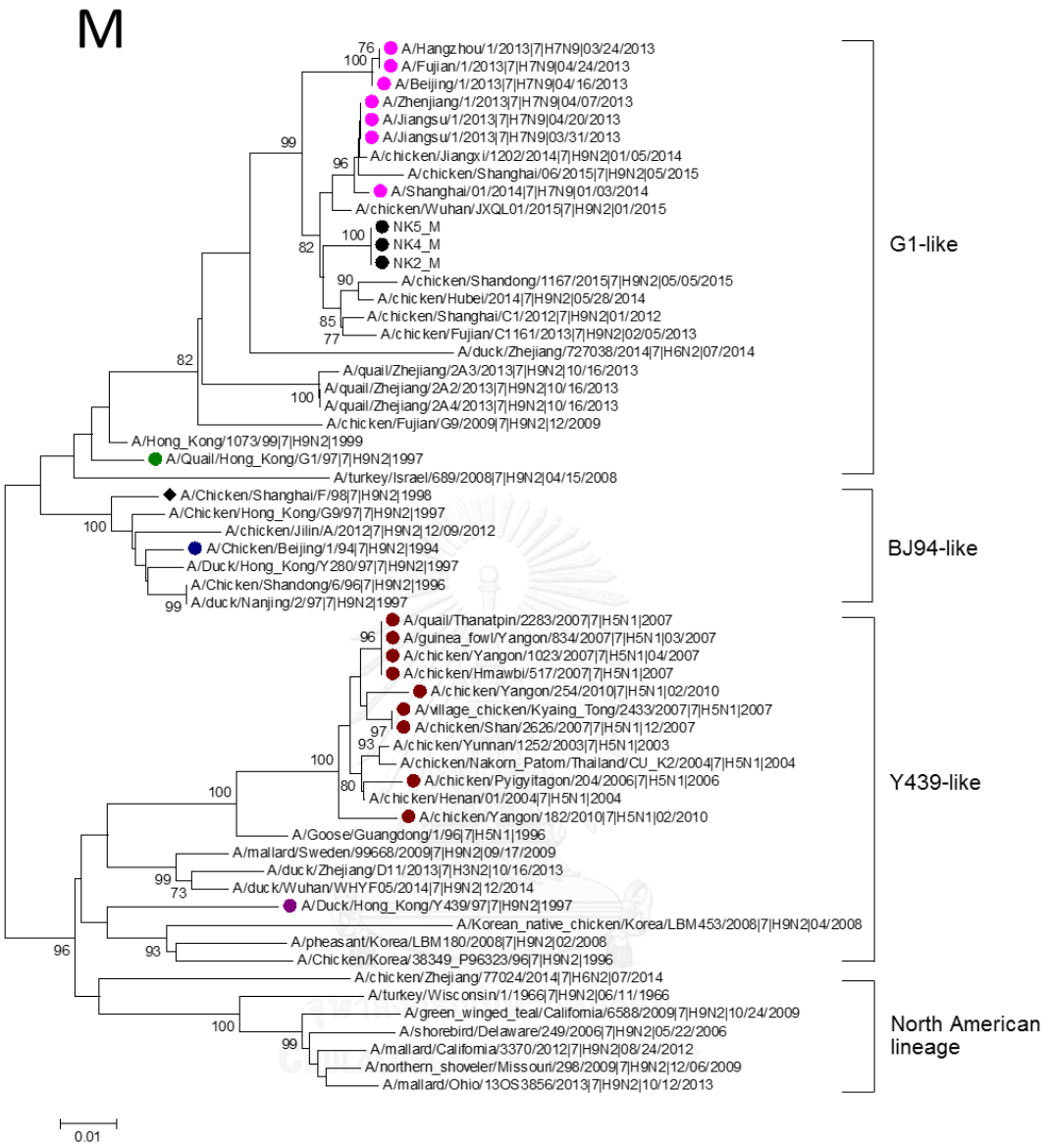

Figure 22: Phylogenetic analysis of the M genes of all three H9N2 isolates

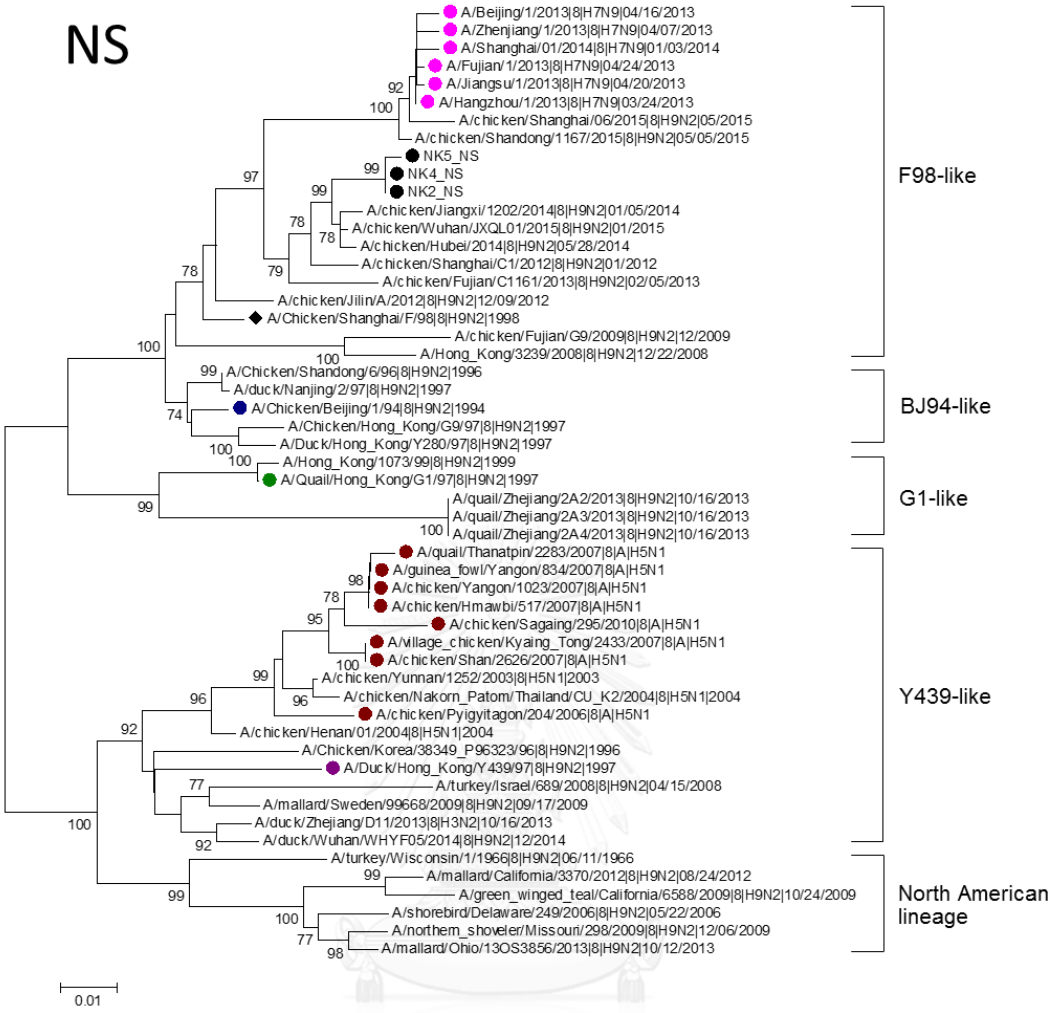

Figure 23: Phylogenetic analysis of the NS genes of all three H9N2 isolates

CHAPTER V DISCUSSION

Prior to this study, the reports of influenza A virus infections in animals in Myanmar were focused only on avian influenza. No report or surveillance has ever been done in other animal species. Even with the avian influenza, the information is not comprehensive and mainly represents the IAV subtype H5. Most of all, there is limited information referring to the genesis of influenza A virus infections in animals in Myanmar. Therefore, this study was focusing on the surveillances of influenza A virus infections in poultry and dogs. Our results provided the information of molecular characterization of low pathogenic avian influenza virus subtype H9N2 in Myanmar. Moreover, it also provided the information of canine influenza in Myanmar.

In this study, the occurrence of influenza A virus in poultry in LBMs by virus isolation was 0.23%; (3/1,278), and it was higher than the previous occurrences of influenza A virus in LBMs (0% in 2006), (0.07% in 2007), and (0% in 2008) in Myanmar (Kyaw et al., 2008). However, the occurrence in this study was lower than the occurrences of influenza A virus in LBMs in China (6.3%) during 2013-2014 (Luan et al., 2016) and Vietnam (5.8%) during 2014 (Thuy et al., 2016). The occurrence in this study was very low, compared to the occurrences of influenza A virus in LBMs from Bangladesh (23%) during 2011 (Negovetich et al., 2011) and Cambodia (45%) during 2013 (Horm et al., 2016). The occurrences in this study was slightly comparable to

influenza virus surveillances in LBMs in Thailand during 2006-2007 (1.3%) 007 (1.3%) (Amonsin et al., 2008) and 2011 (2.39%) (Jairak et al., 2016).

A notable difference in the occurrence of influenza viruses was observed between the Yangon (7.62%) and Namkham (3.86%) LBMs. This can be related to the daily circulation of poultry in each LBM, which is approximately 40,000 in Yangon and 400 in Namkham. High stocking density and close contact among the poultry could be a factor for higher occurrence in Yangon LBM. However, it should be noted that Namkham was regarded as high risk area for influenza in poultry[5] and three IAV subtype H9N2 viruses recovered in this study were collected from Namkham LBM. The sources of poultry were mostly from commercial or medium-scale producers in Yangon LBM, whereas in Namkham small-scale farmers or dealers directly import poultry and poultry products from Ruili (Yunan province) of China). Therefore, in this study, the possible sources of influenza A viruses circulated in the LBM could be from the border area of China.

Among three seasons, a large portion of influenza virus occurrence in poultry (57.53%; 42/73) was detected in the winter. The average temperature in winter is 25°C in Yangon and 20°C in Namkham. It has been reported that the decrease in ambient temperature is related to the occurrence of influenza viruses in animals (Liu et al., 2007; Lowen et al., 2007) and therefore lower temperature can increase the

[5] Emergency surveillance & Emergency Response to AI H7N9 (Value Chain Study in China Border)

chance of IAV occurrence in poultry during winter. In addition, migratory birds fly into Myanmar during winter months and hibernate at the big lakes and wetland areas. Migratory birds are known as the reservoirs of influenza virus and can transmit the virus to domestic poultry (Normile, 2006; Olsen et al., 2006). In other seasons, the occurrence of influenza virus was higher (5.8%) in summer months (March – May 2015) than in rainy months (2.1%) (June-July, 2014 and June – August 2015). It is contradictory with the phenomenon of the influenza virus occurrence in animals that tend to decrease with higher temperature and lower humidity in summer. However, in the history of influenza occurrence in poultry in Myanmar, there were more outbreaks in summer than in rainy season. Influenza outbreaks in poultry were usually reported during winter, transactional period between winter and summer, and early summer months in Myanmar.

In this study, higher occurrence of influenza virus was observed in chickens (7.39%) compared to the ducks (5.26%), which are the natural reservoir of influenza A viruses (Webster et al., 1992). The difference in the number of sampled animals between chickens and ducks (703 vs 380) could be related to higher occurrence in chickens in this study. Among different types of chicken, higher occurrence of influenza virus in broilers (11.64%) than in layer chickens (0.76%) could be associated with short antibody response and low cellular immune response in broiler chickens (Koenen et al., 2002). However, no occurrence of influenza virus was detected in

backyard (village) chickens and it may be partly due to small number of samples (66) collected.

In dogs, the occurrence of influenza virus (21.2%) in this study was similar to previous occurrence reported in dogs in Korea (19.84%) during May 2007 to December 2010 (Song et al., 2012). Lower occurrence of influenza virus was reported in dogs in USA (2.37%) during 2009 – 2012 and in China during 2013 (Chen et al., 2015). A report from Germany stated that the occurrence of influenza virus in dogs was as low as zero (Schulz et al., 2014). However, no previous occurrence of influenza virus in dogs in Myanmar was available for comparison of this study.

By location, the occurrence of influenza virus in dogs was higher in Nay Pyi Taw (24.2%) than in Yangon (3.22%). The difference in occurrence could be influenced by the difference in the number of dogs sampled from each location (172 vs 31). However, to our observations, the difference in the practice of raising and population of stray dogs could be a more reasonable explanation for the higher occurrences in Nay Pyi Taw. A comparative result can be seen in the rainy season of 2014, in which 1 out of 31 dogs from Yangon was positive to canine influenza virus in June 2014, while 5 out of 41 dogs from Nay Pyi Taw were positive in July 2014.

In terms of the seasonal occurrence, more than 79% (34/43) of the canine influenza viruses were detected in winter (December 2014 and January 2015). It should be noted that all samples in winter were collected from dogs in Nay Pyi Taw. In Nay Pyi Taw, the climate is semi-arid and less humid, while it is wet and humid in

Yangon. Therefore, temperature variation is more extreme in Nay Pyi Taw and it is colder than Yangon during winter. The average temperature in December and January in Nay Pyi Taw is 20ºC. The decrease in ambient temperature is known to be associated with the occurrence of influenza viruses in animals, this could be a factor for higher occurrence of influenza virus in dogs from Nay Pyi Taw.

For isolation and subtype identification of influenza A viruses, three influenza viruses were isolated and subtyped as influenza A virus subtype H9N2 in poultry. Interestingly, all three viruses were isolated from chicken. The occurrence of influenza A virus by virus isolation was only 0.23% (3/1278) in this study. However, this was the first report of influenza A virus subtype H9N2 in Myanmar. In dogs, no HA titer was detected with rRT-PCR positive samples (0/43) and no virus could be isolated.

During 8 months of serological surveillance (February – September 2016), highest occurrence of serum antibodies by ELISA (20.8%, 19/91) was observed in February. However, seasonal occurrence of influenza antibodies could not be fully judged because two primary winter months (December and January) were not included in serological surveillance in poultry. Between summer (March – May 2016) and rainy seasons (June – September 2016), the occurrence was slightly higher in summer (13.4%; 28/209 vs 10.3%; 33/321).

In serology, serological surveillance in poultry was conducted only in Yangon LBM. The presence of influenza antibodies in poultry serum (12.8%, 80/621 by ELISA;

1.44%, 9/621 by H5 HI test; 2.8%, 18/621 by H9 HI test indicated that influenza viruses would have been exposed to poultry population in Yangon areas, even though no virus has been isolated from Yangon LBM. However, it was to notice that virological surveillance (June 2014 to May 2015) and serological surveillance (February 2016 to September 2016) in this study were performed in different animals on different timeline, and therefore the circulation of the virus in the seropositive birds could not be ruled out.

In this study, subtype identification was performed only for H5 and H9 subtypes. The difference in the numbers of seropositive poultry between ELISA (80) and HI test (24) suggested the existence of other subtypes in Yangon LBM. In this study, among the birds that were positive to both ELISA and HI test, 5 birds were positive to H5 HI - ELISA combination and 13 were positive to H9 HI - ELISA combination. Interestingly, only 3 out of 18 combinations were ducks, and all three ducks were seropositive to H5 subtype (Table 15). Moreover, the overall seropositive percentage of H5 subtype was remarkably higher in duck than in chickens (3.7% vs 0.8%). On the other hand, H9 subtypes were detected only in chickens. These findings implied the higher susceptibility and early death of chickens when infected with H5 subtypes.

However, a layer chicken positive to all tests (H5, H9 and ELISA) was also observed in this study (Table 15). Moreover, 15 out of 18 dual-test-positive (ELISA and HI test positive) birds were also layer chickens, and seropositive percentage was

highest in layer chickens (38%, 51/134) among the poultry serum (621). This can be related to longer lifespan of layer chickens during which higher exposure of influenza viruses can occur.

In dogs, it was interesting that 6 out of 24 ELISA positive dogs were also positive to RT-PCR detection, and all of them were collected during winter months. Among townships, serum antibody occurrence was observed highest in Pyinmana (19.4%, 13/67). However, higher occurrence in Pyinmana townships could be confounded with the time of sample collection, which was in winter.

In association study, presence of respiratory symptoms, poor body condition scores, season of sampling, and vaccination against influenza were found to be significantly associated with occurrence of influenza virus in dogs. However, it was not possible to judge some hypothesized factors, such as breeds and presence of horse, since all dogs were local breeds and no horse was present in sample collection sites. Moreover, the effect of vaccination could not represent the distribution of influenza antibody-positive samples among the dogs because vaccination was practiced only in a shelter, which was the only shelter in this study located in Yangon. In villages in Nay Pyi Taw, there was no shelter and no practice of vaccination.

For genetic characterization, whole genome of three H9N2 viruses were characterized in this study. The nucleotide sequences of the viruses were compared with other H9N2 isolates from Asia. Phylogenetic analysis showed that all three

isolates had the same origin of Chinese isolates, suggesting that it was introduced from China. However, they were of the origin of BJ94-like (Y280) lineages, whereas viruses circulating in China primarily descend from the BJ/94-like and G1-like genotypes (Xu et al., 2007; Bi et al., 2010; Sun et al., 2010; Zhang et al., 2012).

It was observed that all three isolates carried Q226L substitution at the receptor binding site of HA protein, showing the affinity to human a-2,6-glycan receptors (Matrosovich et al., 1997; Matrosovich et al., 2001). H9N2 viruses with 226L in the HA molecule can replicate efficiently in human airway epithelial cells (Wan and Perez, 2007; Yuan et al., 2015), and could be transmitted by direct contact (Butt et al., 2005; Wan et al., 2008). Although man-to-man infection of H9N2 viruses has yet to be confirmed, transmission of natural isolates via respiratory droplets has been demonstrated in ferrets. Human infection with avian influenza A(H9N2) virus was identified in Bangladesh in 2011 (Shanmuganatham et al., 2013), and in China and Hong Kong in 1999, 2003, and 2013, respectively (Peiris et al., 1999; Butt et al., 2005). L672 on PA genes of all three viruses in this study was an indicator of airborne transmissibility among chicken (Zhang et al., 2014).

Although H9N2 viruses are considered as low pathogenic, attention should be paid since H9N2 are constantly evolving and highly compatible with other subtypes of AIV resulting in novel viruses (To et al., 2013; Chen et al., 2014a). H9N2 viruses were the donors of the internal genes of the H5N1 viruses from Hong Kong in 1997 (Guan et al., 1999) and the novel H7N9 in mainland China in 2013 (Gao et al., 2013;

Kageyama et al., 2013), and the H10N8 Jiangxi province, China (Zhang et al., 2012). Co-infection of H9N2 viruses with bacteria is also common (Bano et al., 2003; Kishida et al., 2004). Furthermore, due to its low pathogenic nature in poultry, transmission to humans is often overlooked. It is therefore of a great public health concern and zoonotic potential of H9N2 IAVs should not be underestimated. The H9N2 IAVs might constitute significant donors of genetic material to emerging human IAVs in future.

Epidemiologically, the spread and evolution of H9N2 viruses are so extensive that they progressively undergo genetic reassortment (Li et al., 2005; Xu et al., 2007; Kimble et al., 2011). Co-circulation of other avian influenza virus subtypes may also result in gradual and complex evolution of H9N2 viruses (Okamatsu et al., 2008).

Mass vaccination is another important factor in the evolution of H9N2 viruses. Antigenic differences between the vaccine strain and prevailing viruses intensify the antigenic drift that cause continuous evolution and drug resistance (Fazel et al., 2014; Wei et al., 2016). Despite widespread vaccination programs (Li et al., 2005), H9N2 viruses are still regularly isolated from birds, many vaccinated poultry flocks have been reported of influenza virus infections (Sun et al., 2012). In this study, the amantadine resistance (S31N substitution on M2 protein) was observed in all three isolates. Therefore, all three H9N2 viruses from Namkham could be the result of either vaccination or direct transmission of the viruses from infected poultry.

This study also reminded that the importance of live-bird markets should not be underestimated. All three isolates characterized in this study were obtained from chickens in live-bird markets with poor sanitary condition. It is widely accepted that live-bird markets can harbor different kinds of pathogen. They are usually heavily contaminated and are regarded as the major source of the spread of influenza viruses (Indriani et al., 2010; Kang et al., 2015). With viruses disseminating in LBM, mixing and exchange of gene segments can occur, and transmission between humans and poultry can take place (Indriani et al., 2010; Chen et al., 2014b). Studies revealed that live-bird markets were closely associated with the H5N1, H7N9, and H9N2 infections in humans (Guan et al., 1999; Li et al., 2003; Chen et al., 2013).

Finally, this study has provided the information related to avian and canine influenza viruses in poultry and dogs in Myanmar. Epidemiological findings from this study and genesis of H9N2 viruses in poultry in Myanmar will be useful for the authorities and general public in need. Additional information on molecular characteristics of H9N2 viruses will be available in influenza database for scientific communities.

CHAPTER VI: CONCLUSION AND RECOMMENDATION

In this study, the status of IAV infection and antibodies to IAV was investigated in poultry and dogs in Namkham, Yangon, and Nay Pyi Taw areas, Myanmar. Poultry from two LBM and dogs from four townships were surveyed. The surveillance lasted for 28 months. In poultry, a total of 1,278 swab samples were collected from ducks (n=380), chicken (n=703), and environmental sites (n=195). It was noted that, 630 swab (315 oropharyngeal and 315 clocal swab) samples were collected from LBM in Yangon, while 648 swab samples (453 oropharyngeal swabs and 195 environmental swabs) were collected from LBM in Namkham. For serology, the serum samples were collected from LBM in Yangon. A total of 621 blood samples were collected from ducks (n=132) and chickens (n=489) in the LBM. In dogs, nasal swabs (n=203) and serum samples (n=203) were collected from 203 dogs in 14 villages in Yangon and Nay Pyi Taw areas. A total of 31 dogs from Yangon and 172 dogs from Nay Pyi Taw were sampled. The number of real-time RT-PCR positive swab samples was 73 in poultry and 43 in dogs. From dogs, no virus could be isolated. Three viruses were isolated from chicken swab samples from Namkham LBM. The viruses were identified as influenza IAV subtype H9N2 and characterized by whole genome sequencing. Genetic characterization of all three viruses showed that the viruses belong to the H9 lineage of 9.4.2.5. The serological surveillance showed that serum antibodies were detected in 80 poultry and 24 dogs by ELISA. Out of 80 ELISA-

positive poultry, 9 birds showed the presence of antibodies to H5N1 and 15 birds showed antibodies to H9N2 subtypes.

This study was carried out to examine the current status and previous exposure of influenza A virus infection in poultry and dogs in Myanmar. A 28-month-long cross-sectional study was conducted for 3 objectives. For the first objective, the occurrence of avian and canine influenza infection in poultry and dogs were determined. For the second objective, the outcomes of genetic analysis and diversity of influenza A viruses in poultry was revealed. However, unfortunately, no genetic characterization could be accomplished in dogs. For the third objectives, the epidemiological findings on associated factors of influenza A virus infection in dogs were reported.

Findings from this study indicated that LBM is an important source of infection for influenza viruses in poultry in Myanmar. Several predisposing factors, such as slaughtering of poultry in LBM, flow of blood on the grounds, and improper disposal of offal and feathers, were observed during the study. Live poultry of different species and different sources are also retained closely in LBM. Furthermore, biosecurity in humans is very low in LBM in this study. Food stalls are amid the live poultry and people are buying and eating from them. Glove-wearing is also not practiced. Therefore, some control measures should be taken to reduce the possible contamination in LBM, especially to minimize the potential poultry-to-human transmission. Although all-in-all-out practice would not be feasible,

especially in Yangon LBM, restriction of the entry of poultry trucks into the LBM at night times and disinfection are recommended tasks before the start of next day.

Another reminder from this study was the importance of proper quarantine check points in border areas. As influenza A virus subtype H9N2 isolated in this study were recovered from chicken in Namkham LBM, in which poultry are mainly collected from Ruili (Yunan province) of China, it is important to be aware of the cross-border trade. An outbreak is very likely to occur if new subtypes are evolved and transmitted to Myanmar in such less-restricted condition.

Serological findings revealed that some poultry and dogs in this study have been previously exposed to influenza A virus infections. High antibodies occurrence in serum and low viral titer in swab indicated the need of consecutive surveillances and monitoring programs to investigate the virus more efficiently.

The difference between high numbers of ELISA positives and low numbers of subtype-specific antibodies (H5 and H9) in poultry serum indicated the circulation of other subtypes in LBM. Since LBM are widely known as the sources of IAVs infection in humans, it would be interesting to conduct a parallel sero-surveillance in both humans and poultry to represent human-animal interface of the infection.

In conclusion, this study provided additional information on avian influenza in poultry and canine influenza in dogs in Myanmar. However, due to some constraints, including the limitation of time and re-entry permission in Myanmar, some works in this study, such as monthly sample collection and serological test for multiple

subtypes, have not been accomplished to full extent. More regularly scheduled molecular surveillance is therefore necessary for further exploration into the influenza A virus infection in animals in Myanmar. Finding from this study indicated that influenza A virus infection is evolving in animals in Myanmar and it will be helpful for the preparedness of avian and canine influenza in Myanmar in future.

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APPENDIX

APPENDIX A

Protocol for RNA extraction

Step 1: 600 µl of RAV1 buffer was mixed with 150 µl of allantoic fluid and incubated at 70ºC for 5 minutes.

Step 2: 600 µl of ethanol was added to the tube and mixed by vortexing for 15 seconds.

Step 3: 700 µl of lysed samples was added to Nucleospin® RNA virus columns in collection tubes (2ml) and centrifuged for 1 minute at 8,000 x g.

Step 4: 500 µl of RAW buffer was added to the Nucleospin® RNA virus columns in collection tubes (2ml), centrifuged for 1 minute at 8,000 x g, and flow-through was discarded.

Step 5: 600 µl of RAV3 buffer was added to the Nucleospin® RNA virus columns, centrifuged for 1 minute at 8,000 x g, and flow-through was discarded with collection tubes.

Step 6: 200 µl of RAV3 buffer was added to the Nucleospin® RNA virus columns in new collection tubes (2ml) and centrifuged for 3 minutes at 11,000 x g.

Step 7: 50 µl of RNase-free water (preheated to 70ºC) was added to the Nucleospin® RNA virus columns in sterile microcentrifuge tube (1.5 ml), incubated for 2 minutes at room temperature, and centrifuged for 1 minute at 11,000 x g.

APPENDIX B

Protocol for real-time RT-PCR (rRT-PCR)

Reagents	**Volume**
RNA	4 µl
10 µM forward primers	0.5 µl
10 µM reverse primers	0.5 µl
2.5 µM M64 probe	0.5 µl
2x Master mix	6.25 µl
Superscript III	0.25 µl
Distilled water	0.42 µl
50 µM $MgSO_4$	0.08 µl
Final volume	**12.5 µl**

PCR condition for real-time RT-PCR

Step 1: 50ºC for 30 minutes

Step 2: 95ºC for 15 minutes followed by 50 cycles of amplification

Step 3: 95ºC for 15 seconds

Step 4: 60ºC for 30 seconds

APPENDIX C

Protocol for cDNA synthesis

First step

Reagents	**Volume**
RNA	5 µl
Random primer	5 µl
(incubated at 70ºC for 15 min; at 4ºC for 5 min)	

Second step

Distilled water	3.7 µl
5x cDNA buffer	4 µl
2.5 mM $MgCl_2$	2 µl
0.5 mM dNTP	1 µl
RNase inhibitor	0.3 µl
ImProm-II™ Reverse Transcriptase	1 µl
Final volume	**22 µl**

PCR condition for cDNA synthesis

Step 1: 25ºC for 5 minutes

Step 2: 42ºC for 60 minutes

Step 3: 72ºC for 15 minutes

Step 4: 95ºC for pause

APPENDIX D

Protocol for PCR subtyping

Reagents	**Volume**
cDNA	0.5 µl
10 µM forward primers	0.5 µl
10 µM reverse primers	0.5 µl
2xKAPA Taq Master Mix	5 µl
Distilled water	3.5 µl
Final volume	**10 µl**

PCR condition for real-time RT-PCR

Step 1: 94ºC for 2 minutes

Step 2: 94ºC for 30 seconds

Step 3: 50ºC for 30 seconds (for HA) and 50ºC for 30 seconds (for NA)

Step 4: 72ºC for 30 seconds

(Step 2 to 4 repeated for 40 cycles)

Step 5: 72ºC for 7 minutes

Step 6: 72ºC for pause

APPENDIX E

Letters and codes of amino acids

Amino acid	3 letter	1 letter	Codes
Alanine	Ala	A	GCT, GCC, GCA, GCG
Arginine	Arg	R	CGT, CGC, CGA, CGG, AGA, AGG
Asparagine	Asn	N	AAT, AAC
Aspartic acid	Asp	D	GAT, GAC
Cysteine	Cys	C	TGT, TGC
Glutamic acid	Glu	E	GAA, GAG
Glutamine	Gln	Q	CAA, CAG
Glycine	Gly	G	GGT, GGC, GGA, GGG
Histidine	His	H	CAT, CAC
Isoleucine	Ile	I	ATT, ATC, ATA
Leucine	Leu	L	CTT, CTC, CTA, CTG, TTA, TTG
Lysine	Lys	K	AAA, AAG
Methionine	Met	M	ATG
Phenylalanine	Phe	F	TTT, TTC
Proline	Pro	P	CCT, CCC, CCA, CCG
Serine	Ser	S	TCT, TCC, TCA, TCG, AGT, AGC
Threonine	Thr	T	ACT, ACC, ACA, ACG
Tryptophan	Trp	W	TGG
Tyrosine	Tyr	Y	TAT, TAC
Valine	Val	V	GTT, GTC, GTA, GTG

APPENDIX F

Nucleotide sequences

A/Chicken/Myanmar/NK-2/2015(H9N2)

Segment 1: Polymerase basic protein 2 (PB2) gene

Nucleotides: 2,280 bp

GenBank: KY115364

ATGGAAAGAATAAAAGAACTAAGAGATTTGATGTCACAGTCTCGCACTCGCGAGATACTGACAAAAACAACTG
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GGTGGAACAGAAATGGACCAACGACAAGCACAGTCCACTATCCAAAGGTCTATAAAACCTATTTTGAAAAGGT
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ATAAACCCAGGCCATGCAGATCTTAGTGCTAAGGAAGCACAAGATGTCATCATGGAGGTCGTATTCCCAAACG
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AAAGATTCAATGGTCCCAAGATCCTACAATGCTATACAATAAGATGGAATTTGAACCCTTTCAATCCCTAGTGC
CTAAAGCTGTCAGAGGCCAATATAGTGGGTTCGTAAGGGTTCTATTCCAACAGATGCGTGACGTACTGGGGAC
ATTTGACACTGTCCAAATAATAAAGCTATTACCATTTGCAGCAGCCCCGCCGGAGCAGAGTAGGATGCAGTTC
TCTTCTCTAACTGTGAATGTGAGAGGTTCAGGAATGAGAATAGTTGTGAGAGGCAATTCTCCTGTGTTCAACTA
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AACAGCAGGAGTGGAATCTGCAGTATTGAGGGGGTTTCTGATTCTGGGCAAAGAGGACAAGAGATATGGGCCA
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TGGTGTTGGTAATGAAACGGAAACGGGACTCTAGCATACTTACTGACAGTCAGACAGCGACCAAAAGGATTCG
GATGGCCATCAATTAA

Segment 2: Polymerase basic protein 1 (PB1) gene

Nucleotides: 2,274 bp

GenBank: KY115365

ATGGATGTCAATCCGACTTTACTCTTCTTGAAAGTGCCAGTGCAAAATGCTGTAAGTACCACGTTCCCTTATAC
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Segment 3: Polymerase acidic protein (PA) gene

Nucleotides: 2,151 bp

GenBank: KY115366

ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATCGTCGAGCTTGCGGAAAAGGCAATGAAAGAATATG
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GAAGGTGTGCAGAACCTTACTGGCAAAATCTGTATTCAATAGCCTATATGCATCTCCACAACTCGAGGGGTTC
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AACTCCTTCCTCACACATGCACTAAGATAG

Segment 4: Hemagglutinin (HA) gene

Nucleotides: 1,683 bp

GenBank: KY115367

ATGGAAACAGTATCACTAATAACTATACTACTAGTAGCAACAGTAAGCAATGCAGATAAAATCTGCATCGGCTA
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ATTCGGAACGGGACCTACAACAGGAGGAAATATCAAGAGGAGTCAAAATTAGAAAGACAGAAAATAGAGGGGG
TCAAGCTGGAATCTGAAGGAACTTACAAAATCCTCACCATTTATTCGACTGTCGCCTCATCTCTTGTGATTGCA
ATGGGGTTTGCTGCCTTCTTGTTCTGGGCCATGTCCAATGGGTCTTGCAGATGCAACATTTGTATATAA

Segment 5: Nucleocapsid protein (NP) gene

Nucleotides: 1,497 bp

GenBank: KY115368

ATGGCGTCTCAAGGCACCAAACGATCCTATGAACAGATGGAAACTGGTGGAGAACGCCAGAATG
CTACTGAAATCAGGGCATCTGTCGGAAGAATGGTTAGCGGCATTGGGAGATTCTACATACAGAT
GTGTACAGAACTCAAACTCAGTGACAATGAAGGGAGGCTGATCCAGAACAGTATAACAATAGAG
AGAATGGTACTCTCTGCATTTGATGAAAGAAGGAACAGATACCTGGAAGAGCACCCCAGTGCGG
GAAAGGACCCGAAGAAAACTGGAGGTCCAATTTACCGAAGAAGAGACGGGAAGTGGGTGAGAG
AGCTGATCCTGTATGACAAGGAGGAAATCAGGAGAATTTGGCGTCAGGCGAACAATGGAGAGG
ACGCAACTGCTGGTCTTACCCATCTAATGATATGGCATTCCAACCTGAATGATGCCACATATCA
GAGAACTAGAGCTCTTGTGCGTACTGGAATGGACCCCAGGATGTGCTCTCTGATGCAAGGATCA
ACTCTCCCGAGGAGATCTGGAGCTGCAGGAGCAGCAGTAAAGGGGATAGGGACGATGGTGATG
GAGCTGATTCGGATGATAAAACGAGGGATAAACGACCGGAATTTCTGGAGAGGCGAAAATGGAA
GAAGAACAAGAGTTGCATACGAGAGGATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAG
CACAAAGAGCAATGGTGGATCAAGTGCGAGAGAGCAGAAATCCTGGAAATGCTGAAATAGAAGA
TCTCATTTTTCTGGCAAGGTCTGCACTCATTCTGAGAGGATCTGTGGCCCATAAGTCCTGCTTG
CCTGCTTGTGTGTACGGACTTGCAGTGGCCAGTGGATATGACTTTGAGAGAGAAGGATACTCCC
TGGTTGGAATAGACCCTTTCCGTCTACTTCAAAACAGCCAGGTTTTTAGTCTAATTAGACCAAAT
GAGAACCCAGCGCACAAGAGTCAACTGGTGTGGATGGCATGCCACTCTGCAGCATTTGAGGACC

TTAGAGTCTCAAGTTTCATCAGAGGGACAAGAATGGTCCCAAGAGGACAGTTATCCACCAGAGG
GGTTCAAATCGCTTCAAATGAGAACATGGAAGCAATGGACTCCAACACTCTTGAACTGAGAAGT
AGGTATTGGGCTATAAGAACCAGGAGCGGAGGGAACACCAACCAACAGAGGGCATCTGCGGGA
CAAATTAGCGTTCAACCCACTTTCTCGGTACAGAGAAATCTCCCCTTCGAAAGAGCGACCATTA
TGGCAGCTTTTACAGGAAATACTGAGGGTAGAACGTCTGACATGAGGACTGAAATCATAAGAAT
GATGGAAAGTGCAAGACCAGAAGATGTGTCATTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGA
CGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTC
GGAGACAATGCAGAGGAGTATGACAATTAA

Segment 6: Neuraminidase (NA) gene

Nucleotides: 1,401 bp

GenBank: KY115369

ATGAATCCAAACCAGAAGATAATAGCAATTGGCTCTGTTTCTCTGATAATTGCGATAATATGTCTCCTCATGCA
GATTGCCATCTTAACAACGACTATGACACTGCATTTCAGGCAGAATGAATGCAGTAACCCACCGAATAATCAA
GTGATGCCATGTGAACCAATCATATTAGAGAGGAACACAGTGCACTTGAATAGCACTATCATAGAGAGGGAAA
TTTGTCCCAAAGTAGCAAACTACAAGAATTGGTCAAAACCACAATGTCAAATTACAGGGTTCGCTCCTTTCTCA
AAGGACAACTCAATTAGGCTTTCTGCAGGTGGGGATATCTGGGTGACAAGAGAACCTTATGTCTCATGCAGTC
TCGACAAATGTTATCAATTTGCACTTGGGCAGGGAACCACCCTGAAAAACAAGCACTCAAATGGCACTACTCA
TGATAGAACTCCTCACAGAACTCTTTTAATGAATGAGTTAGGTGTCCCATTTCATTTGGGAACCAAACAAGTGT
GCATGGCATGGTCTAGTTCAAGCTGCTATGATGGAAAAGCATGGTTACACATTTGTGTTACTGGGGATGATAA
AAATGCTACTGCTAGTATCATCTATGATGGGATGCTTGTTGACAGCATTGGTTCATGGTCCAAAAACATCCTCA
GAACTCAGGAGTCAGAATGCGTTTGTATCAATGGAACTTGTGCAGTGGTAATGACTGATGGAAGTGCATCAGG
AAAGGCAGACACTAGGGTATTATTCATAAGAGAAGGGAAAATTATAAATATTAGCCCACTGTCAGGAAGTGCT
CAGCACGTTGAGGAATGCTCTTGTTATCCTCGGTATCCTGAAGTTAGATGTGTCTGCAGAGACAATTGGAAGG
GCTCCAATAGGCCCATTCTATATATAAATATGGCTGATTATAGCATTGAGTCCAGTTATGTGTGCTCAGGACTT
GTCGGCGACACACCAAGAAATGATGATAGCTCCAGCAGTAGCAACTGCAGAGACCCTAACAACGAAAGAGGGG
CCCCAGGAGTGAAAGGGTGGGCCTTTGATGACGGGAATGATGTTTGGATGGGACGGACAATCAAAAATGGTTC
ACGATCAGGTTATGAGACTTTTAGGGTCGTTAATGGTTGGACCATGGCTAATTCAAAGTCACAGATAAATAGG
CAAGTCATAGTTGACAGTGACGACTGGTCTGGGTATTCTGGCATCTTCTCTGTTGAAGGCAAAAAATGTATCAA
CAGGTGTTTTTATGTGGAGTTGATAAGAGGGAGACCACAGGAACCCAGAGTGTGGTGGACCTCAAATAGCATC

ATTGTATTCTGTGGAACCTCAGGTACATATGGAACAGGCTCATGGCCTGATGGAGCGAATATCAACTTCATGC
CTGTATAA

Segment 7: Matrix protein (M) gene

Nucleotides: 968 bp

GenBank: KY115370

ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATTCCATCAGGCCCCCTCAAAGCCGAGATCG
CGCAGAGACTTGAGGATGTTTTTGCAGGGAAGAACGCAGATCTCGAGGCTCTCATGGAGTGGATAAAGACAAG
ACCAATCCTGTCACCTCTGACTAAGGGGATTTTAGGGTTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGA
CTGCAGCGTAGACGTTTTGTCCAAAACGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAAGGCAGTTA
AATTATACAAGAAACTGAAGAGGGAAATGACATTTCATGGAGCAAAGGAAGTTGCACTCAGTTACTCAACTGG
TGCGCTTGCCAGCTGCATGGGTCTCATATACAACAGGATGGGGACAGTGACCGCAGAAGGGGCTCTTGGACTA
GTATGTGCCACTTGTGAGCAAATTGCTGATGCTCAACATCGATCCCACAGGCAGATGGCGACTACTACCAACC
CACTAATTAGGCATGAGAATAGAATGGTACTGGCCAGCACTACGGCTAAGGCTATGGAGCAGATGGCTGGATC
AAGTGAACAGGCAGCGGAAGCCATGGAAGTCGCAAGCCAGGCTAGGCAAATGGTGCAGGCTATGAGAACAGT
CGGGACTCACCCTAACTCCAGTACAGGTCTAAAGGATGATCTTATTGAAAATTTGCAGGCTTACCAGAACCGG
ATGGGAGTGCAACTGCAGCGGTTCAAGTGATCCCCTCGTTGTTGCAGCTAACATTATTGGGATATTGCACTTG
ATATTGTGGATTCTTGATCGTCTTTTCTTCAAATGCATTTATCGTCGCTTTAAATACGGTTTGAAAAGAGGGCC
TTCTACGGAAGGAATGCCTGAGTCTATGAGGGAAGAATATCGGCAGGAACAGCAGAATGCTGTGGATGTTGAC
GATGGTCATTTTGTCAACA

Segment 8: Nonstructural protein (NS) gene

Nucleotides: 852 bp

GenBank: KY115371

ATGGATTCCAACACTGTGTCAAGCTTCCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAACGGTTTGCAGACC
AAGAACTGGGTGATGCCCCATTTCTAGACCGGCTTCGCCGAGACCAGAAGTCCCTGAGAGGAAGAAGCAGCAC
CCTTGGTCTGGACATCAGAACAGCAACTCGTGAAGGAAAGCATATAGTGGAGCGAATTTTGGAGGAAGAGTCT
GACGAAGCATTTAAAATGACTATTGCTTCAGTGCCAGCTCCACGCTATCTAACTGACATGACTCTTGAAGAAAT
GTCAAGAGATTGGTTAATGCTCATACCCAAGCAGAAAGTGACAGGGTCCCTTTGCATTAGAATGGACCAAGCA
ATAGTGGACAAAACCATCACATTGAAAGCAAATTTCAGTGTAATTTTCAATCGATTGGAAGCCCTAATACTACT

TAGAGCTTTTACGGATGAAGGAGCAATAGTGGGCGAAATCTCACCATTACCTTCTCTTCCAGGACATACTGAC
AAGGATGTCAAAAATGCAATTGAGGTCCTCATCGGAGGATTTGAATGGAATGATAACACAGTTCGAGTCTCTG
AAGCTCTACAGAGATTCGCTTGGAGAAGCAGCGATGAGGATGGGAGACCTCCACTCTCTCCAAAGTAGGAACG
GGAAATGGAGAGAACAATTAAGCCAGAAGTTCGAAGAAATAAGATGGTTGATTGAAGAAGTACGACATAGGTT
AAAGATTACAGAGAATAGCTTTGAACAAATAACTTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAG
AGATAAGAACTTTCTCGTTTCAGCTTATTTAATGATAAAAAACACC

A/Chicken/Myanmar/NK-4/2015 (H9N2)

Segment 1: Polymerase basic protein 2 (PB2) gene

Nucleotides: 2,280 bp

GenBank: KY115372

ATGGAAAGAATAAAAGAACTAAGAGATTTGATGTCACAGTCTCGCACTCGCGAGATACTGACAAAAACAACTG
TAGACCATATGGCAATAATCAAGAAATACACATCGGGAAGACAGGAGAAGAATCCTGCCCTTAGGATGAAGTG
GATGATGGCGATGAAATATCCAATTACAGCAGACAAAAGGATAATGGAGATGATCCCGGAAAGGAATGAGCAA
GGTCAGACCCTTTGGAGCAAGACAAATGATGCTGGATCAGACAGAGTGATGGTGTCACCTCTGGCTGTGACGT
GGTGGAACAGAAATGGACCAACGACAAGCACAGTCCACTATCCAAAGGTCTATAAAACCTATTTTGAAAAGGT
CGAAAGACTAAAACATGGGACCTTCGGCCCCGTTCACTTCCGAAATCAGGTTAAAATACGCCGCAGGGTCGAC
ATAAACCCAGGCCATGCAGATCTTAGTGCTAAGGAAGCACAAGATGTCATCATGGAGGTCGTATTCCCAAACG
AAGTTGGAGCCAGAATATTAACATCAGAGTCACAGTTAACGATAACCAAGGAAAAGAAGGAGGAGCTTCAGGA
CTGCAAAATTGCCCCTTTAATGGTGGCTTACATGTTGGAGAGGGAACTAGTTCGCAAAACAAGATTTCTACCA
GTAGCTGGGGGGACAAGCAGCGTGTATATCGAAGTATTACATTTGACCCAAGGAACCTGCTGGGAGCAAATGT
ACACACCGGGAGGGGAAGTGAGAAATGATGATGTTGATCAGAGTTTAATTATTGCTGCTAGAAATATTGTTAG
AAGAGCAACAGTGTCAGCAGACCCGTTGGCTTCGCTCTTGGAGATGTGCCATAGTACACAGATTGGCGGAGTT
AGGATGGTTGACATACTTAGACAAAACCCAACAGAAGAACAGGCTGTGGATATATGTAAGGCAGCAATGGGCC
TAAGGATCAGTTCATCCTTCAGCTTTGGAGGTTTCACTTTCAAAAGGACAAGTGGGTCATCTGTCAAAAGGGA
AGAAGAAGTGCTCACAGGCAACCTCCAAACATTGAAAATAAGAGTACATGAAGGATATGAGGAATTCACAATG
GTTGGGCGAAGAGCAACAGCCATTCTAAGGAAAGCAACCAGAAGACTGATCCAATTGATAGTGAGTGGGAAAG
ACGAGCAATCAATTGCCGAGGCAATCATAGTGGCAATGGTATTCTCACAAGAGGATTGTATGATAAAGGCAGT
GAGAGGTGATTTGAACTTTGTCAACAGAGCAAACCAACGGCTAAATCCCATGCATCAACTCCTGAGGCATTTC
CAAAAGGATGCTAAGGTCCTGTTTCAAAACTGGGGAATTGAACCCATTGACAATGTAATGGGGATGATCGGAA
TATTGCCTGACATGACCCCCAGCACAGAGATGTCATTGAGAGGAGTGAGAGTTAGTAAAATGGGAGTAGATGA

ATATTCCAGTACTGAGAGAGTGGTCGTGAGTATTGATCATTTCTTGAGGGTTCGAGACCAGAGGGGAAACGTA
CTCCTGTCTCCCGAAGAGGTTAGTGAAACACAGGGAACAGAAAAGCTGACTATAACATATTCATCGTCCATGA
TGTGGGAGATCAATGGTCCGGAATCAGTGCTAGTTAACACATATCAATGGATCATTAGAAATTGGGAAACTGT
AAAGATTCAATGGTCCCAAGATCCTACAATGCTATACAATAAGATGGAATTTGAACCCTTTCAATCCCTAGTGC
CTAAAGCTGTCAGAGGCCAATATAGTGGGTTCGTAAGGGTTCTATTCCAACAGATGCGTGACGTACTGGGGAC
ATTTGACACTGTCCAAATAATAAAGCTATTACCATTTGCAGCAGCCCCGCCGGAGCAGAGTAGGATGCAGTTC
TCTTCTCTAACTGTGAATGTGAGAGGTTCAGGAATGAGAATAGTTGTGAGAGGCAATTCTCCTGTGTTCAACTA
CAACAAGGCAACCAAGAGGCTTACGGTGCTTGGGAAGGATGCAGGTGCATTGATGGAAGACCCAGATGAGGG
AACAGCAGGAGTGGAATCTGCAGTATTGAGGGGGTTTCTGATTCTGGGCAAAGAGGACAAGAGATATGGGCCA
GCATTGAGCATCAACGAATTGAGCAATCTTGCGAAAGGAGAGAAGGCTAATGTTTTGATAGGGCAAGGAGACG
TGGTGTTGGTAATGAAACGGAAACGGGACTCTAGCATACTTACTGACAGTCAGACAGCGACCAAAAGGATTCG
GATGGCCATCAATTAA

Segment 2: Polymerase basic protein 1 (PB1) gene

Nucleotides: 2,274 bp

GenBank: KY115373

ATGGATGTCAATCCGACTTTACTCTTCTTGAAAGTGCCAGTGCAAAATGCTGTAAGTACCACGTTCCCTTATAC
TGGCGACCCTCCATACAGCCATGGAACAGGAACAGGATACACCATGGACACAGTCAACAGAACACATAAATAC
TCAGAAAAAGGAAAGTGGACAACGAACACAGAGACTGGAGCACCCCAGCTCAATCCGATTGATGGACCGTTGC
CTGAGGACAATGAGCCGAGTGGGTATGCACAAACGGATTGTGTATTGGAAGCAATGGCTTTCCTTGAAGAATC
TCATCCAGGGATTTTTGAAAACTCGTGTCTCGAAACGATGGAAATTGTTCAGCAAACAAGAGTGGATAAACTG
ACCCAAGGCCGCCAGACCTATGACTGGACATTGAACAGAAACCAGCCGGCTGCTACTGCATTGGCCAACACTA
TAGAGGTATTCAGATCGAATGGCCTGACAGCTAATGAATCAGGAAGGTTGATTGATTTCCTCAAGGATGTGGT
GGATTCAATGGATAAGGGAGAAATGGAGATTACAACACATTTCCAGAGGAAGAGGAGAGTGAGGGACAACATG
ACCAAGAAAATGGTCACACAAAGAACAATAGGAAAGAAAAAACAAAGACTGAACAAAAAGAGCTACCTAATAA
GAGCATTGACATTGAACACAATGACAAAGGATGCTGAAAGAGGCAAGTTAAAAAGGAGGGCAATCGCAACACC
CGGGATGCAAATCAGAGGATTCGTGTATTTTGTAGAAGCACTAGCGAGGAGCATCTGTGAGAAACTTGAGCAA
TCTGGCCTCCCTGTTGGAGGGAATGAGAAGAAAGCTAAATTGGCAAATGTTGTGAGGAAGATGATGACTAATT
CACAAGACACAGAGCTCTCCTTCACAATTACTGGAGACAACACCAAATGGAATGAGAATCAAAACCCCCGGAT
GTTTCTAGCAATGATAACATACATCACAAGAAACCAGCCAGAATGGTTTAGAAATGTCTTAAGCATTGCTCCTA
TAATGTTCTCAAACAAAATGGCGAGGTTAGGAAAAGGATACATGTTCGAAAGTAAGAGTATGAAGCTACGAAC
ACAAGTACCAGCAGAAATGCTAGCGAATATTGATCTGAAATACTTCAACAAATCAACAAGAGAGAAGATCGAG

AAAATAAGGCCTCTACTAATAGATGGCACAGCCTCATTGAGTCCTGGGATGATGATGGGTATGTTCAATATGTT
GAGTACAGTATTAGGAGTTTCAATTCTGAATCTCGGGCAAAAGAAGTACACCAAAACCACATATTGGTGGGAC
GGACTCCAATCCTCAGATGACTTCGCCCTCATAGTGAATGCACCGAATCATGAGGGAATACAGGCAGGAGTGG
ACAGGTTCTATAGAACCTGCAAATTAGTTGGGATAAACATGAGCAAGAAGAAATCCTACATAAATCGAACAGG
AACATTCGAATTCACAAGCTTTTTCTACCGCTATGGATTCGTGGCTAACTTCAGTATGGAGTTGCCCAGTTTTG
GAGTGTCTGGGATTAATGAGTCAGCTGACATGAGCATTGGTGTTACAGTAATAAAGAACAATATGATAAACAA
CGATCTTGGACCAGCAACAGCCCAGATGGCCCTTCAGCTATTTATCAAAGACTACAGATACACATACCGATGT
CACAGGGGTGATACGCAAATTCAAACGAGGAGAGCGTTCGAGCTGAAGAAACTGTGGGAGCAGACCCGGTCG
AAGGCAGGACTGCTGGTTTCTGATGGAGGGCCAAACCTGTACAATATTCGGAACCTCCACATCCCAGAGGTCT
GCTTGAAATGGGAATTGATGGATGAAGACTACCAAGGCAGGCTGTGTAATCCTATGAACCCGTTTGTCAGTCA
TAAGGAAATTGATTCCGTCAACAATGCGGTGGTGATGCCAGCTCATGGCCCAGCTAAAAGCATGGAATATGAT
GCCGTTGCAACTACACATTCATGGATCCCTAAAAGGAATCGCTCCATTCTCAACACCAGCCAAAGAGGGATTC
TTGAGGATGAACAGATGTACCAGAAGTGCTGCAACCTATTCGAAAAGTTCTTCCCCAGCAGTTCATATCGGAG
GCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGATGCACGAATTGACTTCGAA
TCTGGAAGGATTAAGAAAGAAGAGTTTGCCGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGC
AAAAATAG

Segment 3: Polymerase acidic protein (PA) gene

Nucleotides: 2151 bp

GenBank: KY115374

ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATCGTCGAGCTTGCGGAAAAGGCAATGAAAGAATATG
GGGAAGATCCGAAGATCGAAACAAACAAATTCGCATCAATATGCACACACTTAGAAGTCTGCTTCATGTACTC
TGATTTCCACTTCATCGACGAACGGGGCGAATCAACTATTATAGAATCTGGCGATCCAAATGCGTTGTTGAAA
CATCGATTTGAAATAATTGAAGGGAGAGACCGAACAATGGCCTGGACAGTGGTGAATAGTATTTGCAACACCA
CAGGAGTCGAAAAACCTAAATTCCTCCCGGATCTGTATGACTACAAGGAAAACCGATTCATTGAAATTGGAGT
GACGAGGAGGGAAGTCCACATATATTACCTAGAGAAAGCCAATAAAATAAAATCCGAGAAGACACACATCCAC
ATTTTCTCATTCACTGGAGAAGAGATGGCCACCAAAGCAGATTACACTCTTGACGAAGAAAGCAGGGCAAGAA
TCAAAACCAGGTTGTTCACCATAAGGCAGGAAATGGCCAGCAGGGGCCTATGGGATTCCTTTCGTCAGTCCGA
AAGAGGCGAAGAAACAATTGAAGAAAGATTTGAAATCACAGGAACCATGCGCAGGCTTGCCGACCAAAGTCTC
CCACCGAACTTCTCCAGCCTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGG
GCAAGCTTTCTCAAATGTCAAAAGAAGTGAACGCCAGAATCGAGCCATTTCTAAAGACAACACCACGCCCTCT
CAGATTGCCTAATGGCCCTCCCTGTTCTCAGCGGTCGAAATTCTTGCTGATGGATGCTCTGAAATTAAGCATTG

AGGACCCGAGCCACGAAGGGGAGGGGATACCTCTTTATGATGCGATCAAATGCATGAAAACGTTCTTCGGGTG
GAAAGAGCCCAACATTATCAAACCACATGAGAAAGGCATAAACCCCAACTATCTCCTGACTTGGAAGCAGGTG
CTAGCAGAACTTCAGGACATTGAAAATGAAGAGAAAATTCCAAGGACAAAGAACATGAAGAAAACAAGCCAAT
TAAAGTGGGCACTCGGTGAAAACATGGCACCGGAGAAGGTGGACTTTGAGGATTGCAAAGATGTCAACGACCT
GAAACAGTACGACAGTGATGAGCCAGAGCCCAGATCACTAGCATGTTGGATCCAGAACGAATTCAACAAGGCA
TGTGAATTGACTGATTCAAGCTGGGTAGAGCTTGATGAAATAGGAGATGATGTTGCCCCAATCGAACACATTG
CAAGCATGAGACGGAACTATTTTACAGCAGAGGTGTCCCACTGCAGGGCTACTGAATACATAATGAAGGGAGT
GTACATAAATACAGCTTTGCTCAATGCATCTTGTGCAGCCATGGATGACTTTCAACTGATTCCAATGATAAGTA
AATGCAGAACCAAAGAAGGAAGACGGAAAACAAACCTATATGGATTCATTATAAAAGGAAGATCTCATTTGAG
GAATGATACCGATGTGGTTAACTTTGTAAGTATGGAATTTTCCCTTACTGACCCAAGGTTGGAACCACATAAAT
GGGAAAAGTATTGTGTTCTTGAAATAGGGGATATGCTCCTGCGAACTGCAGTAGGCCAGGTGTCAAGACCCAT
GTTTCTGTATGTGAGAACCAATGGGACCTCCAAGATCAAGATGAAATGGGGTATGGAAATGAGACGCTGCCTT
CTTCAATCCCTCCAACAGATTGAGAGCATGATTGAAGCTGAATCCTCCGTCAAAGAGAAAGACCTGACCAAAG
AATTCTTTGAAAACAAATCAGAAACATGGCCAATTGGAGAATCACCTAAGGGAGTGGAGGAAGGCTCCATCGG
GAAGGTGTGCAGAACCTTACTAGCAAAATCTGTATTCAATAGCCTATATGCATCTCCACAACTCGAGGGGTTCT
CAGCTGAATCGAGAAAACTGCTGCTCATTGTTCAGGCGCTTAGGGATAACCTGGAACCTGGAACCTTCGATCT
TGAAGGGCTATATGAAGCAATCGAGGAGTGCCTGATTAATGATCCCTGGGTTTTGCTTAATGCATCTTGGTTCA
ACTCCTTCCTCACACATGCACTAAGATAG

Segment 4: Hemagglutinin (HA) gene

Nucleotides: 1,683 bp

GenBank: KY115375

ATGGAAACAGTATCACTAATAACTATACTACTAGTAGCAACAGTAAGCAATGCAGATAAAATCTGCATCGGCTA
TCAATCAACAAACTCTACAGAAACTGTGGACACACTAACAGAAACCAATGTCCCTGTGACACATGCCAAAGAA
TTGCTCCACACAGAGCATAATGGGATGCTGTGTGCAACAAGCTTGGGACAACCTCTTATTTTAGACACCTGCA
CCATTGAAGGGCTAATCTATGGCAATCCTTCTTGTGATCTATCGCTGGAAGGAAGAGAATGGTCCTATATCGT
CGAGAGACCATCAGCTGTTAACGGATTGTGTTACCCCGGGAATGTAGAAAATCTAGAAGAGCTAAGGTCACTT
TTTAGTTCTGCTAGGTCTTATCAAAGAATCCAGATTTTCCCAGACACAATCTGGAATGTGTCTTACGATGGGAC
AAGCACAGCATGCTCAGGTTCATTCTACAGGAGCATGAGATGGTTGACTCGAAAGAACGGCGAATACTCTATC
CAAGACGCCCAATACACAAATAATCAAGGGAAGAACATTCTTTTCATGTGGGGCATAAATCACCCACCCACCG
ATACTACGCAGATAAATCTGTACACGAGAACCGACACAACAACGAGTGTGGCAACAGAAGAAATAAATAGGAT
CTTCAAACCATTGATAGGACCAAGGCCCCTTGTCAACGGTTTGATGGGAAGAATTGATTATTATTGGTCGGTTT

TGAAACCAGGTCAAACACTGCGAATAAAATCTGATGGGAATCTAATAGCTCCATGGTTTGGACACATTCTTTCA
GGAGAGAGCCACGGAAGAATTCTGAAGACTGATCTAAAAAGGGGTAGCTGCACAGTGCAATGTCAGACAGAGA
AAGGTGGCTTAAACACAACATTGCCATTCCAAAATGTAAGTAAGTATGCATTTGGAAACTGCTCAAAATACATT
GGCATAAAGAGTCTCAAACTTGCAGTTGGTCTGAGGAATGTGCCTTCTAGATCTAGTAGAGGACTATTCGGGG
CCATAGCAGGGTTTATAGAGGGAGGTTGGTCAGGACTAGTCGCTGGTTGGTATGGGTTCCAGCATTCAAATGA
CCAAGGGGTTGGTATGGCAGCAGATAGAGACTCAACTCAAAAGGCAATTGATAAAATAACATCCAAAGTGAAT
AATATAGTCGACAAAATGAACAAGCAATATGAAATCATCGATCATGAATTCAGTGAGGTAGAAACTAGACTTAA
CATGATCAATAATAAGATCGATGATCAAATCCAGGATATATGGGCATATAATGCAGAATTGCTAGTTCTGCTTG
AAAACCAGAAAACACTCGATGAGCATGATGCAAATGTAAACAATCTATATAATAAAGTAAAGAGGGCGTTGGG
TTCCAATGCGGTAGAAGATGGGAAAGGATGTTTCGAGCTATACCACAAATGTGATGACCAATGCATGGAGACA
ATTCGGAACGGGACCTACAACAGGAGGAAATATCAAGAGGAGTCAAAATTAGAAAGACAGAAAATAGAGGGGG
TCAAGCTGGAATCTGAAGGAACTTACAAAATCCTCACCATTTATTCGACTGTCGCCTCATCTCTTGTGATTGCA
ATGGGGTTTGCTGCCTTCTTGTTCTGGGCCATGTCCAATGGGTCTTGCAGATGCAACATTTGTATATAA

Segment 5: Nucleocapsid protein (NP) gene

Nucleotides: 1,466 bp

GenBank: KY115376

ATGGCGTCTCAAGGCACCAAACGATCCTATGAACAGATGGAAACTGGTGGAGAACGCCAGAATGCTACTGAAA
TCAGGGCATCTGTCGGAAGAATGGTTAGCGGCATTGGGAGATTCTACATACAGATGTGTACAGAACTCAAACT
CAGTGACAATGAAGGGAGGCTGATCCAGAACAGTATAACAATAGAGAGAATGGTACTCTCTGCATTTGATGAA
AGAAGGAACAGATACCTGGAAGAGCACCCCAGTGCGGGAAAGGACCCGAAGAAAACTGGAGGTCCAATTTACC
GAAGAAGAGACGGGAAGTGGGTGAGAGAGCTGATCCTGTATGACAAGGAGGAAATCAGGAGAATTTGGCGTC
AGGCGAACAATGGAGAGGACGCAACTGCTGGTCTTACCCATCTAATGATATGGCATTCCAACCTGAATGATGC
CACATATCAGAGAACTAGAGCTCTTGTGCGTACTGGAATGGACCCCAGGATGTGCTCTCTGATGCAAGGATCA
ACTCTCCCGAGGAGATCTGGAGCTGCAGGAGCAGCAGTAAAGGGGATAGGGACGATGGTGATGGAGCTGATT
CGGATGATAAAACGAGGGATAAACGACCGGAATTTCTGGAGAGGCGAAAATGGAAGAAGAACAAGAGTTGCAT
ACGAGAGGATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGAGCAATGGTGGATCAAGTGCG
AGAGAGCAGAAATCCTGGAAATGCTGAAATAGAAGATCTCATTTTTCTGGCAAGGTCTGCACTCATTCTGAGA
GGATCTGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCAGTGGCCAGTGGATATGACTTTG
AGAGAGAAGGATACTCCCTGGTTGGAATAGACCCTTTCCGTCTACTTCAAAACAGCCAGGTTTTTAGTCTAATT
AGACCAAATGAGAACCCAGCGCACAAGAGCCAACTGGTGTGGATGGCATGCCACTCTGCAGCATTTGAGGACC
TTAGAGTCTCAAGTTTCATCAGAGGGACAAGAATGGTCCCAAGAGGACAGTTATCCACCAGAGGGGTTCAAAT

CGCTTCAAATGAGAACATGGAAGCAATGGACTCCAACACTCTTGAACTGAGAAGTAGGTATTGGGCTATAAGA
ACCAGGAGCGGAGGGAACACCAACCAACAGAGGGCATCTGCGGGACAAATTAGCGTTCAACCCACTTTCTCGG
TACAGAGAAATCTCCCCTTCGAAAGAGCGACCATTATGGCAGCTTTTACAGGAAATACTGAGGGTAGAACGTC
TGACATGAGGACTGAAATCATAAGAATGATGGAAAGTGCAAGACCAGAAGATGTGTCATTCCAGGGGCGGGGA
GTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTT
ATTTCTT

Segment 6: Neuraminidase (NA) gene

Nucleotides: 1,401 bp

GenBank: KY115377

ATGAATCCAAACCAGAAGATAATAGCAATTGGCTCTGTTTCTCTGATAATTGCGATAATATGTCTCCTCATGCA
GATTGCCATCTTAACAACGACTATGACACTGCATTTCAGGCAGAATGAATGCAGTAACCCACCGAATAATCAA
GTGATGCCATGTGAACCAATCATATTAGAGAGGAACACAGTGCACTTGAATAGCACTATCATAGAGAGGGAAA
TTTGTCCCAAAGTAGCAAACTACAAGAATTGGTCAAAACCACAATGTCAAATTACAGGGTTCGCTCCTTTCTCA
AAGGACAACTCAATTAGGCTTTCTGCAGGTGGGGATATCTGGGTGACAAGAGAACCTTATGTCTCATGCAGTC
TCGACAAATGTTATCAATTTGCACTTGGGCAGGGAACCACCCTGAAAAACAAGCACTCAAATGGCACTACTCA
TGATAGAACTCCTCACAGAACTCTTTTAATGAATGAGTTAGGTGTCCCATTTCATTTGGGAACCAAACAAGTGT
GCATGGCATGGTCTAGTTCAAGCTGCTATGATGGAAAAGCATGGTTACACATTTGTGTTACTGGGGATGATAA
AAATGCTACTGCTAGTATCATCTATGATGGGATGCTTGTTGACAGCATTGGTTCATGGTCCAAAAACATCCTCA
GAACTCAGGAGTCAGAATGCGTTTGTATCAATGGAACTTGTGCAGTGGTAATGACTGATGGAAGTGCATCAGG
AAAGGCAGACACTAGGGTATTATTCATAAGAGAAGGGAAAATTATAAATATTAGCCCACTGTCAGGAAGTGCT
CAGCACGTTGAGGAATGCTCTTGTTATCCTCGGTATCCTGAAGTTAGATGTGTCTGCAGAGACAATTGGAAGG
GCTCCAATAGGCCCATTCTATATATAAATATGGCTGATTATAGCATTGAGTCCAGTTATGTGTGTTCAGGACTT
GTCGGCGACACACCAAGAAATGATGATAGCTCCAGCAGTAGCAACTGCAGAGACCCTAACAACGAAAGAGGGG
CCCCAGGAGTGAAAGGGTGGGCCTTTGATGACGGGAATGATGTTTGGATGGGACGGACAATCAAAAATGGTTC
ACGATCAGGTTATGAGACTTTTAGGGTCGTTAATGGTTGGACCATAGCTAATTCAAAGTCACAGATAAATAGG
CAAGTCATAGTTGACAGTGACGACTGGTCTGGGTATTCTGGCATCTTCTCTGTTGAAGGCAAAAAATGTATCAA
CAGGTGTTTTTATGTGGAGTTGATAAGAGGGAGACCACAGGAACCCAGAGTGTGGTGGACCTCAAATAGCATC
ATTGTATTCTGTGGAACCTCAGGTACATATGGAACAGGCTCATGGCCTGATGGAGCGAATATCAACTTCATGC
CTGTATAA

Segment 7: Matrix protein (M) gene

Nucleotides: 968 bp

GenBank: KY115378

ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATTCCATCAGGCCCCCTCAAAGCCGAGATCG
CGCAGAGACTTGAGGATGTTTTTGCAGGGAAGAACGCAGATCTCGAGGCTCTCATGGAGTGGATAAAGACAAG
ACCAATCCTGTCACCTCTGACTAAGGGGATTTTAGGGTTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGA
CTGCAGCGTAGACGTTTTGTCCAAAACGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAAGGCAGTTA
AATTATACAAGAAACTGAAGAGGGAAATGACATTTCATGGAGCAAAGGAAGTTGCACTCAGTTACTCAACTGG
TGCGCTTGCCAGCTGCATGGGTCTCATATACAACAGGATGGGGACAGTGACCGCAGAAGGGGCTCTTGGACTA
GTATGTGCCACTTGTGAGCAAATTGCTGATGCTCAACATCGATCCCACAGGCAGATGGCGACTACTACCAACC
CACTAATTAGGCATGAGAATAGAATGGTACTGGCCAGCACTACGGCTAAGGCTATGGAGCAGATGGCTGGATC
AAGTGAACAGGCAGCGGAAGCCATGGAAGTCGCAAGCCAGGCTAGGCAAATGGTGCAGGCTATGAGAACAGT
CGGGACTCACCCTAACTCCAGTACAGGTCTAAAGGATGATCTTATTGAAAATTTGCAGGCTTACCAGAACCGG
ATGGGAGTGCAACTGCAGCGGTTCAAGTGATCCCCTCGTTGTTGCAGCTAACATTATTGGGATATTGCACTTG
ATATTGTGGATTCTTGATCGTCTTTTCTTCAAATGCATTTATCGTCGCTTTAAATACGGTTTGAAAAGAGGGCC
TTCTACGGAAGGAATGCCTGAGTCTATGAGGGAAGAATATCGGCAGGAACAGCAGAATGCTGTGGATGTTGAC
GATGGTCATTTTGTCAACA

Segment 8: Nonstructural protein (NS) gene

Nucleotides: 853 bp

GenBank: KY115379

ATGGATTCCAACACTGTGTCAAGCTTCCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAACGGTTTGCAGACC
AAGAACTGGGTGATGCCCCATTTCTAGACCGGCTTCGCCGAGACCAGAAGTCCCTGAGAGGAAGAAGCAGCAC
CCTTGGTCTGGACATCAGAACAGCAACTCGTGAAGGAAAGCATATAGTGGAGCGAATTTTGGAGGAAGAGTCT
GACGAAGCATTTAAAATGACTATTGCTTCAGTGCCAGCTCCACGCTATCTAACTGACATGACTCTTGAAGAAAT
GTCAAGAGATTGGTTAATGCTCATACCCAAGCAGAAAGTGACAGGGTCCCTTTGCATTAGAATGGACCAAGCA
ATAGTGGACAAAACCATCACATTGAAAGCAAATTTCAGTGTAATTTTCAATCGATTGGAAGCCCTAATACTACT
TAGAGCTTTTACGGATGAAGGAGCAATAGTGGGCGAAATCTCACCATTACCTTCTCTTCCAGGACATACTGAC
AAGGATGTCAAAAATGCAATTGAGGTCCTCATCGGAGGATTTGAATGGAATGATAACACAGTTCGAGTCTCTG
AAGCTCTACAGAGATTCGCTTGGAGAAGCAGCGATGAGGATGGGAGACCTCCACTCTCTCCAAAGTAGGAACG
GGAAATGGAGAGAACAATTAAGCCAGAAGTTCGAAGAAATAAGATGGTTGATTGAAGAAGTACGACATAGGTT

AAAGATTACAGAGAATAGCTTTGAACAAATAACTTTTATGCAAGCCTTACAACTATTGCTTGAAGTGGAGCAAG
AGATAAGAACTTTCTCGTTTCAGCTTATTTAATGATAAAAAACACCC

A/Chicken/Myanmar/NK-5/2015 (H9N2)

Segment 1: Polymerase basic protein 2 (PB2) gene

Nucleotides: 2190 bp

GenBank: KY115380

ATGGAAAGAATAAAAGAACTAAGAGATTTGATGTCACAGTCTCGCACTCGCGAGATACTGACAAAAACAACTG
TAGACCATATGGCAATAATCAAGAAATACACATCGGGAAGACAGGAGAAGAATCCTGCCCTTAGGATGAAGTG
GATGATGGCGATGAAATATCCAATTACAGCAGACAAAAGGATAATGGAGATGATCCCGGAAAGGAATGAGCAA
GGTCAGACCCTTTGGAGCAAGACAAATGATGCTGGATCAGACAGAGTGATGGTGTCACCTCTGGCTGTGACGT
GGTGGAACAGAAATGGACCAACGACAAGCACAGTCCACTATCCAAAGGTCTATAAAACCTATTTTGAAAAGGT
CGAAAGACTAAAACATGGGACCTTCGGCCCCGTTCACTTCCGAAATCAGGTTAAAATACGCCGCAGGGTCGAC
ATAAACCCAGGCCATGCAGATCTTAGTGCTAAGGAAGCACAAGATGTCATCATGGAGGTCGTATTCCCAAACG
AAGTTGGAGCCAGAATATTAACATCAGAGTCACAGTTAACGATAACCAAGGAAAAGAAGGAGGAGCTTCAGGA
CTGCAAAATTGCCCCTTTAATGGTGGCTTACATGTTGGAGAGGGAACTAGTTCGCAAAACAAGATTTCTACCA
GTAGCTGGGGGGACAAGCAGCGTGTATATCGAAGTATTACATTTGACCCAAGGAACCTGCTGGGAGCAAATGT
ACACACCGGGAGGGGAAGTGAGAAATGATGATGTTGATCAGAGTTTAATTATTGCTGCTAGAAATATTGTTAG
AAGAGCAACAGTGTCAGCAGACCCGTTGGCTTCGCTCTTGGAGATGTGCCATAGTACACAGATTGGCGGAGTT
AGGATGGTTGACATACTTAGACAAAACCCAACAGAAGAACAGGCTGTGGATATATGTAAGGCAGCAATGGGCC
TAAGGATCAGTTCATCCTTCAGCTTTGGAGGTTTCACTTTCAAAAGGACAAGTGGGTCATCTGTCAAAAGGGA
AGAAGAAGTGCTCACAGGCAACCTCCAAACATTGAAAATAAGAGTACATGAAGGATATGAGGAATTCACAATG
GTTGGGCGAAGAGCAACAGCCATTCTAAGGAAAGCAACCAGAAGACTGATCCAATTGATAGTGAGTGGGAAAG
ACGAGCAATCAATTGCCGAGGCAATCATAGTGGCAATGGTATTCTCACAAGAGGATTGTATGATAAAGGCAGT
GAGAGGTGATTTGAACTTTGTCAACAGAGCAAACCAACGGCTAAATCCCATGCATCAACTCCTGAGGCATTTC
CAAAAGGATGCTAAGGTCCTGTTTCAAAACTGGGGAATTGAACCCATTGACAATGTAATGGGGATGATCGGAA
TATTGCCTGACATGACCCCCAGCACAGAGATGTCATTGAGAGGAGTGAGAGTTAGTAAAATGGGAGTAGATGA
ATATTCCAGTACTGAGAGAGTGGTCGTGAGTATTGATCATTTCTTGAGGGTTCGAGACCAGAGGGGAAACGTA
CTCCTGTCTCCCGAAGAGGTTAGTGAAACACAGGGAACAGAAAAGCTGACTATAACATATTCATCGTCCATGA
TGTGGGAGATCAATGGTCCGGAATCAGTGCTAGTTAACACATATCAATGGATCATTAGAAATTGGGAAACTGT
AAAGATTCAATGGTCCCAAGATCCTACAATGCTATACAATAAGATGGAATTTGAACCCTTTCAATCCCTAGTGC

CTAAAGCTGTCAGAGGCCAATATAGTGGGTTCGTAAGGGTTCTATTCCAACAGATGCGTGACGTACTGGGGAC
ATTTGACACTGTCCAAATAATAAAGCTATTACCATTTGCAGCAGCCCCGCCGGAGCAGAGTAGGATGCAGTTC
TCTTCTCTAACTGTGAATGTGAGAGGTTCAGGAATGAGAATAGTTGTGAGAGGCAATTCTCCTGTGTTCAACTA
CAACAAGGCAACCAAGAGGCTTACGGTGCTTGGGAAGGATGCAGGTGCATTGATGGAAGACCCAGATGAGGG
AACAGCAGGAGTGGAATCTGCAGTATTGAGGGGGTTTCTGATTCTGGGCAAAGAGGACAAGAGATATGGGCCA
GCATTGAGCATCAACGAATTGAGCAATCTTGCGAAAGGAGAGAAGGCTAATGTTTTGATAGGGCAAGGAGAC

Segment 2: Polymerase basic protein 1 (PB1) gene

Nucleotides: 2274 bp

GenBank: KY115381

ATGGATGTCAATCCGACTTTACTCTTCTTGAAAGTGCCAGTGCAAAATGCTGTAAGTACCACGTTTCCTTATAC
TGGCGACCCTCCATACAGCCATGGAACAGGAACAGGATACACCATGGACACAGTCAACAGAACACATAAATAC
TCAGAAAAAGGAAAGTGGACAACGAACACAGAGACTGGAGCACCCCAGCTCAATCCGATTGATGGACCGTTGC
CTGAGGACAATGAGCCGAGTGGGTATGCACAAACGGATTGTGTATTGGAAGCAATGGCTTTCCTTGAAGAATC
TCATCCAGGGATTTTTGAAAACTCGTGTCTCGAAACGATGGAAATTGTTCAGCAAACAAGAGTGGATAAACTG
ACCCAAGGCCGCCAGACCTATGACTGGACATTGAACAGAAACCAGCCGGCTGCTACTGCATTGGCCAACACTA
TAGAGGTATTCAGATCGAATGGCCTGACAGCTAATGAATCAGGAAGGTTGATTGATTTCCTCAAGGATGTGGT
GGATTCAATGGATAAGGGAGAAATGGAGATTACAACACATTTCCAGAGGAAGAGGAGAGTGAGGGACAACATG
ACCAAGAAAATGGTCACACAAAGAACAATAGGGAAGAAAAAACAAAGACTGAACAAAAAGAGCTACCTAATAA
GGGCATTGACATTGAACACAATGACAAAGGATGCTGAAAGAGGCAAGTTAAAAAGGAGGGCAATCGCAACACC
CGGGATGCAAATCAGAGGATTCGTGTATTTTGTAGAAGCACTAGCGAGGAGCATCTGTGAGAAACTTGAGCAA
TCGGGCCTCCCTGTTGGAGGGAATGAGAAGAAAGCTAAATTGGCAAATGTTGTGAGGAAGATGATGACTAATT
CACAAGACACAGAGCTCTCCTTCACAATTACTGGAGACAACACCAAATGGAATGAGAATCAAAACCCCCGGAT
GTTTCTAGCAATGATAACATACATCACAAGAAACCAGCCAGAATGGTTTAGAAATGTCTTAAGCATTGCTCCTA
TAATGTTCTCAAACAAAATGGCGAGGTTAGGAAAAGGATACATGTTCGAAAGTAAGAGTATGAAGCTACGAAC
ACAAGTACCAGCAGAAATGCTAGCGAATATTGATCTGAAATACTTCAACAAATCAACAAGAGAGAAGATCGAG
AAAATAAGGCCTCTACTAATAGATGGCACAGCCTCATTGAGTCCTGGGATGATGATGGGTATGTTCAATATGTT
GAGTACAGTATTAGGAGTTTCAATTCTGAATCTCGGGCAAAAGAAGTACACCAAAACCACATATTGGTGGGAC
GGACTCCAATCCTCAGATGACTTCGCCCTCATAGTGAATGCACCGAATCATGAGGGAATACAGGCAGGAGTGG
ACAGGTTCTATAGAACCTGCAAATTAGTTGGGATAAACATGAGCAAGAAGAAATCCTACATAAATCGAACAGG
AACATTCGAATTCACAAGCTTTTTCTACCGCTATGGATTCGTAGCTAACTTCAGTATGGAGTTGCCCAGTTTTG
GAGTGTCTGGGATTAATGAGTCAGCTGACATGAGCATTGGTGTTACAGTAATAAAGAACAATATGATAAACAA

CGATCTTGGACCAGCAACAGCCCAGATGGCCCTTCAGCTATTTATCAAAGACTACAGATACACATACCGATGT
CACAGGGGTGATACGCAAATTCAAACGAGGAGAGCGTTCGAGCTGAAGAAACTGTGGGAGCAGACCCGGTCG
AAGGCAGGACTGCTGGTTTCTGATGGAGGGCCAAACCTGTACAATATTCGGAACCTCCACATCCCAGAGGTCT
GCTTGAAATGGGAATTGATGGATGAAGACTACCAAGGCAGGCTGTGTAATCCTATGAACCCGTTTGTCAGTCA
TAAGGAAATTGATTCCGTCAACAATGCGGTGGTGATGCCAGCTCATGGCCCAGCTAAAAGCATGGAATATGAT
GCCGTTGCAACTACACATTCATGGATCCCTAAAAGGAATCGCTCCATTCTCAACACCAGCCAAAGAGGGATTC
TTGAGGATGAACAGATGTACCAGAAGTGCTGCAACCTATTCGAAAAGTTCTTCCCCAGCAGTTCATATCGGAG
GCCAGTTGGAATTTCCAGCATGGTGGAGGCCATGGTGTCTAGGGCCCGAATTGATGCACGAATTGACTTCGAA
TCTGGAAGGATTAAGAAAGAAGAGTTTGCCGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGC
AAAAATAG

Segment 3: Polymerase acidic protein (PA) gene

Nucleotides: 2151 bp

GenBank: KY115382

ATGGAAGACTTTGTGCGACAATGCTTCAATCCAATGATCGTCGAGCTTGCGGAAAAGGCAATGAAAGAATATG
GGGAAGATCCGAAGATCGAAACAAACAAATTCGCATCAATATGCACACACTTAGAAGTCTGCTTCATGTACTC
TGATTTCCACTTCATCGACGAACGGGGCGAATCAACTATTATAGAATCTGGCGATCCAAATGCGTTGTTGAAA
CATCGATTTGAAATAATTGAAGGGAGAGACCGAACAATGGCCTGGACAGTGGTGAATAGTATTTGCAACACCA
CAGGAGTCGAAAAACCTAAATTCCTCCCGGATCTGTATGACTACAAGGAAAACCGATTCATTGAAATTGGAGT
GACGAGGAGGGAAGTCCACATATATTACCTAGAGAAAGCCAATAAAATAAAATCCGAGAAGACACACATCCAC
ATTTTCTCATTCACTGGAGAAGAGATGGCCACCAAAGCAGATTACACTCTTGACGAAGAAAGCAGGGCAAGAA
TCAAAACCAGGTTGTTCACCATAAGGCAGGAAATGGCCAGCAGGGGCCTATGGGATTCCTTTCGTCAGTCCGA
GAGAGGCGAAGAAACAATTGAAGAAAGATTTGAAATCACCGGAACCATGCGCAGGCTTGCCGACCAAAGTCTC
CCACCGAAATTCTCCAGCCTTGAAAACTTTAGAGCCTATGTGGATGGATTCGAACCGAACGGCTGCATTGAGG
GCAAGCTTTCTCAAATGTCAAAAGAAGTGAACGCCAGAATCGAGCCATTTCTAAAGACAACACCACGCCCTCT
CAGATTGCCTAATGGCCCTCCCTGTTCTCAGCGGTCGAAATTCTTGCTGATGGATGCTCTGAAATTAAGCATTG
AGGACCCGAGCCACGAAGGGGAGGGGATACCTCTTTATGATGCGATCAAATGCATGAAAACGTTCTTCGGGTG
GAAAGAGCCCAACATTATCAAACCACATGAGAAAGGCATAAACCCCAACTATCTCCTGACTTGGAAGCAGGTG
CTAGCAGAACTTCAGGACATTGAAAATGAAGAGAAAATTCCAAGGACAAAGAACATGAAGAAAACAAGCCAAT
TAAAGTGGGCACTCGGTGAAAACATGGCACCGGAGAAGGTGGACTTTGAGGATTGCAAAGATGTCAACGACCT
GAAACAGTACGACAGTGATGAGCCAGAGCCCAGATCACTAGCATGTTGGATCCAGAACGAATTCAACAAGGCA
TGTGAATTGACTGATTCAAGCTGGGTAGAGCTTGATGAAATAGGAGATGATGTTGCCCCAATCGAACACATTG

CAAGCATGAGACGGAACTATTTTACAGCAGAGGTGTCCCACTGCAGGGCTACTGAATACATAATGAAGGGAGT
GTACATAAATACAGCTTTGCTCAATGCATCTTGTGCAGCCATGGATGACTTTCAACTGATTCCAATGATAAGTA
AATGCAGAACCAAAGAAGGAAGACGGAAAACAAACCTATATGGATTCATTATAAAAGGAAGATCTCATTTGAG
GAATGATACCGATGTGGTTAACTTTGTAAGTATGGAATTTTCCCTTACTGACCCAAGGTTGGAACCACATAAAT
GGGAAAAGTATTGTGTTCTTGAAATAGGGGATATGCTCCTGCGAACTGCAGTAGGCCAGGTGTCAAGACCCAT
GTTTCTGTATGTGAGAACCAATGGGACCTCCAAGATCAAGATGAAATGGGGTATGGAAATGAGACGCTGCCTT
CTTCAATCCCTCCAACAGATTGAGAGCATGATTGAAGCTGAATCCTCCGTCAAAGAGAAAGACCTGACCAAAG
AATTCTTTGAAAACAAATCAGAAACATGGCCAATTGGAGAATCACCTAAGGGAGTGGAGGAAGGCTCCATCGG
GAAGGTGTGCAGAACCTTACTAGCAAAATCTGTATTCAATAGCCTATATGCATCTCCACAACTCGAGGGGTTCT
CAGCTGAATCGAGAAAACTGCTGCTCATTGTTCAGGCGCTTAGGGATAACCTGGAACCTGGAACCTTCGATCT
TGAAGGGCTATATGAAGCAATCGAGGAGTGCCTGATTAATGATCCCTGGGTTTTGCTTAATGCATCTTGGTTCA
ACTCCTTCCTCACACATGCACTAAGATAG

Segment 4: Hemagglutinin (HA) gene

Nucleotides: 1683 bp

GenBank: KY115383

ATGGAAACAGTATCACTAATAACTATACTACTAGTAGCAACAGTAAGCAATGCAGATAAAATCTGCATCGGCTA
TCAATCAACAAACTCTACAGAAACTGTGGACACACTAACAGAAACCAATGTCCCTGTGACACATGCCAAAGAA
TTGCTCCACACAGAGCATAATGGGATGCTGTGTGCAACAAGCTTGGGACAACCTCTTATTTTAGACACCTGCA
CCATTGAAGGGCTAATCTATGGCAATCCTTCTTGTGATCTATCGCTGGAAGGAAGAGAATGGTCCTATATCGT
CGAGAGACCATCAGCTGTTAACGGATTGTGTTACCCCGGGAATGTAGAAAATCTAGAAGAGCTAAGGTCACTT
TTTAGTTCTGCTAGGTCTTATCAAAGAATCCAGATTTTCCCAGACACAATCTGGAATGTGTCTTACGATGGGAC
AAGCACAGCATGCTCAGGTTCATTCTACAGGAGCATGAGATGGTTGACTCGAAAGAACGGCGAATACTCTATC
CAAGACGCCCAATACACAAATAATCAAGGGAAGAACATTCTTTTCATGTGGGGCATAAATCACCCACCCACCG
ATACTACGCAGATAAATCTGTACACGAGAACCGACACAACAACGAGTGTGGCAACAGAAGAAATAAATAGGAT
CTTCAAACCATTGATAGGACCAAGGCCCCTTGTCAACGGTTTGATGGGAAGAATTGATTATTATTGGTCGGTTT
TGAAACCAGGTCAAACACTGCGAATAAAATCTGATGGGAATCTAATAGCTCCATGGTTTGGACACATTCTTTCA
GGAGAGAGCCACGGAAGAATTCTGAAGACTGATCTAAAAAGGGGTAGCTGCACAGTGCAATGTCAGACAGAGA
AAGGTGGCTTAAACACAACATTGCCATTCCAAAATGTAAGTAAGTATGCATTTGGAAACTGCTCAAAATACATT
GGCATAAAGAGTCTCAAACTTGCAGTTGGTCTGAGGAATGTGCCTTCTAGATCTAGTAGAGGACTATTCGGGG
CCATAGCAGGGTTTATAGAGGGAGGTTGGTCAGGACTAGTCGCTGGTTGGTATGGGTTCCAGCATTCAAATGA
CCAAGGGGTTGGTATGGCAGCAGATAGAGACTCAACTCAAAAGGCAATTGATAAAATAACATCCAAAGTGAAT

AATATAGTCGACAAAATGAACAAGCAATATGAAATCATCGATCATGAATTCAGTGAGGTAGAAACTAGACTTAA
CATGATCAATAATAAGATCGATGATCAAATCCAGGATATATGGGCATATAATGCAGAATTGCTAGTTCTGCTTG
AAAACCAGAAAACACTCGATGAGCATGATGCAAATGTAAACAATCTATATAATAAAGTAAAGAGGGCGTTGGG
TTCCAATGCGGTAGAAGATGGGAAAGGATGTTTCGAGCTATACCACAAATGTGATGACCAATGCATGGAGACA
ATTCGGAACGGGACCTACAACAGGAGGAAATATCAAGAGGAGTCAAAATTAGAAAGACAGAAAATAGAGGGGG
TCAAGCTGGAATCTGAAGGAACTTACAAAATCCTCACCATTTATTCGACTGTCGCCTCATCTCTTGTGATTGCA
ATGGGGTTTGCTGCCTTCTTGTTCTGGGCCATGTCCAATGGGTCTTGCAGATGCAACATTTGTATATAA

Segment 5: Nucleocapsid protein (NP) gene

Nucleotides: 1497 bp

GenBank: KY115384

ATGGCGTCTCAAGGCACCAAACGATCCTATGAACAGATGGAAACTGGTGGAGAACGCCAGAATGCTACTGAAA
TCAGGGCATCTGTCGGAAGAATGGTTAGCGGCATTGGGAGATTCTACATACAGATGTGTACAGAACTCAAACT
CAGTGACAATGAAGGGAGGCTGATCCAGAACAGTATAACAATAGAGAGAATGGTACTCTCTGCATTTGATGAA
AGAAGGAACAGATACCTGGAAGAGCACCCCAGTGCGGGAAAGGACCCGAAGAAAACTGGAGGTCCAATTTACC
GAAGAAGAGACGGGAAGTGGGTGAGAGAGCTGATCCTGTATGACAAGGAGGAAATCAGGAGAATTTGGCGTC
AGGCGAACAATGGAGAGGACGCAACTGCTGGTCTTACCCATCTAATGATATGGCATTCCAACCTGAATGATGC
CACATATCAGAGAACTAGAGCTCTTGTGCGTACTGGAATGGACCCCAGGATGTGCTCTCTGATGCAAGGATCA
ACTCTCCCGAGGAGATCTGGAGCTGCAGGAGCAGCAGTAAAGGGGATAGGGACGATGGTGATGGAGCTGATT
CGGATGATAAAACGAGGGATAAACGACCGGAATTTCTGGAGAGGCGAAAATGGAAGAAGAACAAGAGTTGCAT
ACGAGAGGATGTGCAACATCCTCAAAGGGAAATTCCAAACAGCAGCACAAAGAGCAATGGTGGATCAAGTGCG
AGAGAGCAGAAATCCTGGAAATGCTGAAATAGAAGATCTCATTTTTCTGGCAAGGTCTGCACTCATTCTGAGA
GGATCTGTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCAGTGGCCAGTGGATATGACTTTG
AGAGAGAAGGATACTCCCTGGTTGGAATAGACCCTTTCCGTCTACTTCAAAACAGCCAGGTTTTTAGTCTAATT
AGACCAAATGAGAACCCAGCGCACAAGAGTCAACTGGTGTGGATGGCATGCCACTCTGCAGCATTTGAGGACC
TTAGAGTCTCAAGTTTCATCAGAGGGACAAGAATGGTCCCAAGAGGACAGTTATCCACCAGAGGGGTTCAAAT
CGCTTCAAATGAGAACATGGAAGCAATGGACTCCAACACTCTTGAACTGAGAAGTAGGTATTGGGCTATAAGA
ACCAGGAGCGGAGGGAACACCAACCAACAGAGGGCATCTGCGGGACAAATTAGCGTTCAACCCACTTTCTCGG
TACAGAGAAATCTCCCCTTCGAAAGAGCGACCATTATGGCAGCTTTTACAGGAAATACTGAGGGTAGAACGTC
TGACATGAGGACTGAAATCATAAGAATGATGGAAAGTGCAAGACCAGAAGATGTGTCATTCCAGGGGCGGGGA
GTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATGAAGGATCTT
ATTTCTTCGGAGACAATGCAGAGGAGTATGACAATTAA

Segment 6: Neuraminidase (NA) gene

Nucleotides: 1401 bp

GenBank: KY115385

ATGAATCCAAACCAGAAGATAATAGCAATTGGCTCTGTTTCTCTGATAATTGCGATAATATGTCTCCTCATGCA
GATTGCCATCTTAACAACGACTATGACACTGCATTTCAGGCAGAATGAATGCAGTAACCCACCGAATAATCAA
GTGATGCCATGTGAACCAATCATATTAGAGAGGAACACAGTGCACTTGAATAGCACTATCATAGAGAGGGAAA
TTTGTCCCAAAGTAGCAAACTACAAGAATTGGTCAAAACCACAATGTCAAATTACAGGGTTCGCTCCTTTCTCA
AAGGACAACTCAATTAGGCTTTCTGCAGGTGGGGATATCTGGGTGACAAGAGAACCTTATGTCTCATGCAGTC
TCGACAAATGTTATCAATTTGCACTTGGGCAGGGAACCACCCTGAAAAACAAGCACTCAAATGGCACTACTCA
TGATAGAACTCCTCACAGAACTCTTTTAATGAATGAGTTAGGTGTCCCATTTCATTTGGGAACCAAACAAGTGT
GCATGGCATGGTCTAGTTCAAGCTGCTATGATGGAAAAGCATGGTTACACATTTGTGTTACTGGGGATGATAA
AAATGCTACTGCTAGTATCATCTATGATGGGATGCTTGTTGACAGCATTGGTTCATGGTCCAAAAACATCCTCA
GAACTCAGGAGTCAGAATGCGTTTGTATCAATGGAACTTGTGCAGTGGTAATGACTGATGGAAGTGCATCAGG
AAAGGCAGACACTAGGGTATTATTCATAAGAGAAGGGAAAATTATAAATATTAGCCCACTGTCAGGAAGTGCT
CAGCACGTTGAGGAATGCTCTTGTTATCCTCGGTATCCTGAAGTTAGATGTGTCTGCAGAGACAATTGGAAGG
GCTCCAATAGGCCCATTCTATATATAAATATGGCTGATTATAGCATTGAGTCCAGTTATGTGTGCTCAGGACTT
GTCGGCGACACACCAAGAAATGATGATAGCTCCAGCAGTAGCAACTGCAGAGACCCTAACAACGAAAGAGGGG
CCCCAGGAGTGAAAGGGTGGGCCTTTGATGACGGGAATGATGTTTGGATGGGACGGACAATCAAAAATGGTTC
ACGATCAGGTTATGAGACTTTTAGGGTCGTTAATGGTTGGACCATGGCTAATTCAAAGTCACAGATAAATAGG
CAAGTCATAGTTGACAGTGACGACTGGTCTGGGTATTCTGGCATCTTCTCTGTTGAAGGCAAAAAATGTATCAA
CAGGTGTTTTTATGTGGAGTTGATAAGAGGGAGACCACAGGAACCCAGAGTGTGGTGGACCTCAAATAGCATC
ATTGTATTCTGTGGAACCTCAGGTACATATGGAACAGGCTCATGGCCTGATGGAGCGAATATCAACTTCATGC
CTGTATAA

Segment 7: Matrix protein (M) gene

Nucleotides: 968 bp

GenBank: KY115386

ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATTCCATCAGGCCCCCTCAAAGCCGAGATCG
CGCAGAGACTTGAGGATGTTTTTGCAGGGAAGAACGCAGATCTCGAGGCTCTCATGGAGTGGATAAAGACAAG
ACCAATCCTGTCACCTCTGACTAAGGGGATTTTAGGGTTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGA
CTGCAGCGTAGACGTTTTGTCCAAAACGCCCTAAATGGGAATGGAGACCCAAACAACATGGACAAGGCAGTTA

AATTATACAAGAAACTGAAGAGGGAAATGACATTTCATGGAGCAAAGGAAGTTGCACTCAGTTACTCAACTGG
TGCGCTTGCCAGCTGCATGGGTCTCATATACAACAGGATGGGGACAGTGACCGCAGAAGGGGCTCTTGGACTA
GTATGTGCCACTTGTGAGCAAATTGCTGATGCTCAACATCGATCCCACAGGCAGATGGCGACTACTACCAACC
CACTAATTAGGCATGAGAATAGAATGGTACTGGCCAGCACTACGGCTAAGGCTATGGAGCAGATGGCTGGATC
AAGTGAACAGGCAGCGGAAGCCATGGAAGTCGCAAGCCAGGCTAGGCAAATGGTGCAGGCTATGAGAACAGT
CGGGACTCACCCTAACTCCAGTACAGGTCTAAAGGATGATCTTATTGAAAATTTGCAGGCTTACCAGAACCGG
ATGGGAGTGCAACTGCAGCGGTTCAAGTGATCCCCTCGTTGTTGCAGCTAACATTATTGGGATATTGCACTTG
ATATTGTGGATTCTTGATCGTCTTTTCTTCAAATGCATTTATCGTCGCTTTAAATACGGTTTGAAAAGAGGGCC
TTCTACGGAAGGAATGCCTGAGTCTATGAGGGAAGAATATCGGCAGGAACAGCAGAATGCTGTGGATGTTGAC
GATGGTCATTTTGTCAACA

Segment 8: Nonstructural protein (NS) gene

Nucleotides: 621 bp

GenBank: KY115387

ATGGATTCCAACACTGTGTCAAGCTTCCAGGTAGACTGCTTTCTTTGGCATGTCCGCAAACGGTTTGCAGACC
AAGAACTGGGTGATGCCCCATTTCTAGACCGGCTTCGCCGAGACCAGAAGTCCCTGAGAGGAAGAAGCAGCAC
CCTTGGTCTGGACATCAGAACAGCAACTCGTGAAGGAAAGCATATAGTGGAGCGAATTTTGGAGGAAGAGTCT
GACGAAGCATTTAGAATGACTATTGCTTCAGTGCCAGCTCCACGCTATCTAACTGACATGACTCTTGAAGAAAT
GTCAAGAGATTGGTTAATGCTCATACCCAAGCAGAAAGTGACAGGGTCCCTTTGCATTAGAATGGACCAAGCA
ATAGTGGACAAAACCATCACATTGAAAGCAAATTTCAGTGTAATTTTCAATCGATTGGAAGCCCTAATACTACT
TAGAGCTTTTACGGATGAAGGAGCAATAGTGGGCGAAATCTCACCATTACCTTCTCTTCCAGGACATACTGAC
AAGGATGTCAAAAATGCAATTGAGGTCCTCATCGGAGGATTTGAATGGAATGATAACACAGTTCGAGTCTCTG
AAGCTCTACAGAGATTCGCTTGGAGAAGCAGCGAT

VITA

Mr. Thant Nyi Lin was born in Monywa, Myanmar on October 17, 1980. He got his Bachelor degree in Veterinary Science from the University of Veterinary Science, Yezin, Myanmar in 2005. In 2013, he enrolled in the Degree of Doctor of Philosophy Program in Veterinary Public Health, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University.